ENTRY OF EPSTEIN-BARR VIRUS INTO LYMPHOCYTES AND EPITHELIAL CELLS

By

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DEDICATION

This dissertation is dedicated to my parents, John and Nancy Miller, who have always encouraged all my endeavors and have provided me with love and support throughout all that I have done.

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LIST OF ABBREVATIONS

AF	5-(N-octadecanoyl)aminofluorescein
AIDS	acquired immunodeficiency syndrome
ASF	asialofetuin
a.u.	arbitrary units
ATP	adenosine triphosphate
BL	Burkitt's lymphoma
CMV	cytomegalovirus
CR2	complement receptor 2
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
FACS	fluorescent activated cell sorter
FITC	fluorescein isothiocyanate
HA	hemagglutinin
HIV	human immunodeficiency virus
HN	hemagglutinin-neuramidinase
HSV	herpes simplex virus
Ig	immunoglobulin
I.M.	infectious mononucleosis
LCL	lymphoblastoid cell line
LSM	lymphocyte separation medium
NaN_3	sodium azide
NH_4Cl	ammonium chloride
NPC	nasopharyngeal carcinoma
OHL	oral hairy leukoplakia
SCR	short concensus repeat
SFV	Semliki Forest virus
TPA	12-O-tetradecanoyl phorbol-13-acetate

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Epstein-Barr virus (EBV) is a human herpesvirus which causes infectious mononucleosis and is associated with two cancers, Burkitt's lymphoma and nasopharyngeal carcinoma. To understand the biologic activity of EBV, it is crucial to understand how EBV infects cells, and what viral components are important to this process. Epstein-Barr virus infects two cell types, B lymphocytes and epithelial cells. To examine the early events in virus infection, binding and fusion, we have adapted an assay that measures membrane fusion. Virus membranes were labeled with concentrations of octadecylrhodamine (R_{18}) or 5-(N-octadecanoyl)aminofluorescein (AF) at which fluorescence is self-quenched. The fluorescence of AF is also sensitive to changes in pH. Fusion and mixing of virus and cell membranes was measured in terms of relief of self-quenching and was monitored kinetically.

The assay was used to compare virus fusion with lymphoblastoid cell lines, lymphocytes recently transformed with EBV, normal B lymphocytes and epithelial

cells. Entry of EBV into all cell types occurred independent of exposure to low pH. However, virus fusion with normal and recently transformed lymphocytes occurred from within endocytic vesicles, whereas fusion with lymphoblastoid and epithelial cells occurred at the plasma membrane.

The contribution to fusion made by virus envelope proteins to fusion was studied with monoclonal antibodies that neutralized virus infectivity. Antibody to glycoprotein gp85 inhibited fusion with all cells except epithelial cells. Antibody to glycoprotein gp350, responsible for virus attachment to CR2 on lymphocytes, only partially inhibited virus binding to epithelial cells and the remaining bound virus did not fuse. Soluble CR2 inhibited virus binding to lymphocytes but only partially inhibited binding to epithelial cells.

These studies document clear differences between virus entry into lymphocytes and epithelial cells and suggest that the virus proteins involved in fusion with the two cell types may be distinct.

CHAPTER 1
INTRODUCTION

Discovery of Epstein-Barr Virus

Epstein-Barr virus was discovered by electron microscopy during investigations undertaken with lymphoblastoid cells cultured from a biopsy of an African Burkitt's lymphoma in the early 1960s (Epstein and Barr, 1964). The virus was identified morphologically as a member of the herpesviridae, but extensive virologic investigations proved it to be distinct from any previously known herpesvirus; it could not be transmitted to host cells known to be susceptible to herpes-simplex virus (HSV), cytomegalovirus (CMV), or varicella-zoster virus (VZV) and was given the name Epstein-Barr virus (Epstein et al., 1965). The uniqueness of EBV was confirmed serologically when antisera to known herpesviruses failed to react in immunofluorescence tests with cells carrying the virus (Henle and Henle, 1966). Seroepidemiologic studies established the worldwide distribution of the virus in normal healthy people.

Clinical Manifestations

Burkitt's Lymphoma

In the 1950s Dr. Dennis Burkitt became interested in a children's tumor in Africa that was not only the most common children's tumor in Africa but also more common than all other children's tumors added together (Burkitt, 1987). Also at that

time, M. A. Epstein was in search of a human cancer caused by a virus and became interested in the tumor that Dr. Burkitt described. EBV fulfilled many requirements used to define an oncogenic virus. Virus was present in all tumor cells but not normal tissue from the same patient, patients had extremely high antibody titers to EBV, EBV could immortalize human B lymphocytes in vitro, and EBV was capable of inducing tumors in subhuman primates. However, there could be no simple causal relationship between EBV and BL because the virus was found to infect humans worldwide at a frequency of 90-100% (Evans, 1984). The virus was considered likely to play the role of a cofactor in development of African BL, but additional cellular changes were assumed to occur to create malignant BL cells. The presence of phenotypic differences between EBV-genome containing BL cells and EBV-immortalized nonmalignant cells provided support for this theory. All cell lines derived from BL contained chromosomal translocations (Klein, 1983). The characteristic translocation found in BL is a translocation of chromosome 8 with chromosome 14, but can also involve 2 or 22 (Miyoshi et al., 1981). The c-myc oncogene has been localized to chromosome 8 in humans. The translocations involve the juxtaposition of c-myc with the immunoglobulin heavy chain gene cluster on chromosome 14, the kappa light-chain genes on chromosome 2 and the lambda light-chain genes on chromosome 22 (Lenoir, 1987). It is likely that the c-myc oncogene plays a role in the development of BL, but that the translocation is not induced by EBV. Rather, the appearance of malignant cell clones that have altered c-myc may be facilitated when EBV causes unrestricted proliferation of B cells in cooperation with immunosuppression from persistent malaria infections that are holoendemic in central Africa.

Infectious Mononucleosis

Epstein-Barr virus (EBV) persists in those individuals it infects and induces permanent seroconversion. The virus is transmitted horizontally and primary infection usually takes place in childhood without apparent disease (Henle and Henle, 1979). If the primary infection is delayed until adolescence or young adulthood, which happens at a higher incidence in developed countries, infection leads to infectious mononucleosis (I.M.) in about 50% of cases (Niederman et al., 1970). The first suggestion that EBV was the cause of I.M. came when a laboratory technician (in the laboratory of Drs. W. and G. Henle) who had previously lacked EBV antibodies, seroconverted in the course of I.M.. Her circulating lymphocytes failed to grow in vitro prior to the illness, but gave rise to permanent cultures when collected during the acute phase or during early convalescence (Henle et al., 1968). Following this discovery, a prospective study was conducted at Yale where it was found that all pre-I.M. sera collected lacked antibodies to EBV, while the corresponding acute and convalescence phase sera contained EBV antibodies (Niederman et al., 1968).

The initial step in pathogenesis of any primary infection with EBV, whether symptomatic or not, is entry of the virus into the oropharynx and subsequent replication at that site. The clinical manifestations of EBV-induced I.M. are thought to be caused by a rapid polyclonal T and B cell proliferation. Primary replication of virus occurs in pharyngeal epithelium (Sixbey et al., 1983, 1984) from which circulating B cells are infected and transformed allowing their rapid proliferation (Rickinson et al., 1987; Svedmyr et al., 1984). The symptoms that the I.M. patient experiences are thought to result from the conflict in the immune system as an aggressive T cell response is mounted in order to keep the B cell proliferation in control. Since the

immune system is so important for keeping the abnormal cell proliferation in control, lymphoproliferative disorders can occur in the immunocompromised patient during primary infection or thereafter due to failure to control the persisting latent infection. Particularly at risk are immunosuppressed organ transplant recipients and those with acquired immunodefiency syndrome (AIDS) (Cleary et al., 1986; Fauci, 1988; Hanto et al., 1985; Purtilo, 1985).

Nasopharyngeal Carcinoma

Epstein-Barr virus has also been associated with another human cancer, nasopharyngeal carcinoma (NPC) (Anderson-Anvret et al., 1979; de-The, 1982). The association was initially based on the finding of high antibody titers to EBV in all NPC patients examined (de-The and Zeng, 1987; Henle and Henle, 1976). The antibody levels for viral capsid antigen were unusually high, only paralleled by BL sera, which increased the likelihood of involvement of EBV with the carcinoma. In 1976, EBV DNA was found in all the undifferentiated carcinomas of the nasopharynx studied (Henle and Henle, 1976). Subsequently, EBV DNA has been consistently found in all undifferentiated carcinomas of the nasopharynx and has also been detected in differentiated forms of the carcinoma as well (de-The and Zeng, 1987; Raab-Traub et al., 1987). Nasopharyngeal carcinoma occurs throughout the world, but occurs with a much higher incidence in populations of southern Asia. The high frequency of NPC in the Kwantung Providence of southern China (de-The and Zeng, 1987) suggests that other factors, perhaps genetic or environmental, are acting with EBV in the development of the cancer (Henderson et al., 1976; Klein et al., 1978). Despite considerable effort to identify carcinogenic substances and cultural patterns which might operate as cofactors, no firm identification of such a factor has yet been made.

Oral Hairy Leucoplakia

Epstein-Barr virus is also associated with oral hairy leucoplakia (OHL), a proliferative lesion of the lateral tongue epithelium found in persons infected with HIV (Greenspan et al., 1984). The presence of OHL indicates that patients are severely immunocompromised and has proven to be a valuable prognosticator of the onset of AIDS (Greenspan et al., 1987; Schiodt et al., 1987). Studies of OHL lesions reveal EBV particles within the nucleus, cytoplasm and the intercellular spaces of epithelial cells (Sciubba et al., 1989). In the basal layers, the BZLF1 gene is expressed, which activates the switch from latency into replication. In the upper third of the epithelium, structural proteins and viral envelope components are found (ibid). There is temporary regression of the OHL lesions when the patients are treated with acyclovir, but the lesions recur weeks or months after cessation of acyclovir therapy, indicating that EBV plays an active role in development of the lesions (Resnick et al., 1988).

Description of EBV

Classification and Morphology

Epstein-Barr virus is classified as human herpesvirus 4 and as a member of the gamma herpesvirus subfamily (Roizman, 1982). Morphologically, EBV is indistinguishable from other members of the herpes family. The diameter of the mature virus particle is about 150 to 180 nm. The virus envelope is acquired as the virus buds through the nuclear membrane. The envelope consists of at least five proteins that are encoded by the virus, four of which are glycosylated. The lipid component of the envelope is derived from host cell membrane in which cellular proteins have been replaced by those encoded by the virus (Spear, 1980). Within the

envelope is a nucleocapsid exhibiting isosahedral symmetry which contains 162 capsomeres arranged in hexagonal and pentameric array. An amorphous tegument fills the cavity between the nucleocapsid and the envelope. Inside the nucleocapsid is the core virus particle consisting of core proteins and a large double-stranded deoxyribonucleic acid (DNA) genome of approximately 172,000 base pairs in length (Kieff et al., 1982). The viral mRNAs are translated in the cytoplasm and many of the translational products then return to the nucleus where the nucleocapsid is assembled.

Tropism and Latency

An unique feature of the gamma herpesviruses is their limited host range. All members of this group infect lymphoblastoid cells in vivo and in vitro. The only human member of the group is EBV and was originally identified as having tropism for human B lymphocytes. Other members of the group include Marek's disease virus of chickens and Herpes ateles and Herpes saimiri virus of new world monkeys. These viruses infect T cells (Fleckenstein and Desrosiers, 1982; Nonoyama, 1982). The host range of EBV in vitro is restricted to B lymphocytes of humans and new world primates. EBV establishes latency in these cells and immortalizes them. Latently infected lymphocytes, but not those fully permissive for virus replication, have been demonstrated in vivo. The infected cells retain the complete viral genome and express a restricted set of viral genes necessary to maintain latency (Hayward and Kieff, 1976; Pritchett et al., 1975).

Three types of latently infected cells have been extensively studied, lymphoblastoid cell lines (LCLs), Burkitt-lymphoma cells (BL) and nasopharyngeal cells (NPC). All three types express Epstein-Barr nuclear antigen 1 (EBNA-1), which is

one of the latently transcribed genes that is necessary to maintain the episomal form of the EBV DNA (Fahraesus et al., 1988; Rowe et al., 1987). Additional latently transcribed genes EBNA-2, EBNA-3-6 and latent membrane protein (LMP) are expressed in LCLs, but are down-regulated in BL and NPC cells (Klein, 1989; Rowe et al., 1987).

For a long time it was generally accepted that EBV infected only B cells in vivo. Recently, a second target for EBV, the undifferentiated epithelial cell, has been identified (Greenspan et al., 1985; Lemon et al., 1977; Sixbey et al., 1984, 1987; Wolf et al., 1984). The epithelial cell is permissive for replication and is thought to be the source of virus that is shed in the oropharynx. Cultures of human epithelial cells have been transfected (Grogan et al., 1981) and directly infected in vitro (Sixbey, 1983), but the only cell currently available for studying the virus replication cycle in vitro is the lymphocyte. Lymphocytes latently infected with EBV provide an unique system for studying the biochemistry of herpes virus latency.

The ability of EBV to infect B lymphocytes is initiated by attachment of virus to the 145-kilodalton (kDa) cell membrane glycoprotein, CR2, which also binds the C3d fragment of complement (Fingeroth et al., 1984; Nemerow et al., 1985b). Recently it has been shown that epithelial cells also express a receptor for virus attachment but it is lost during differentiation of the epithelium (Sixbey et al., 1984, 1987; Young et al., 1986). The B cell CR2 receptor is also lost during differentiation to the plasma cell (Tedder et al, 1984). Immunoprecipitation from the surface of epithelial cells with an anti-CR2 antibody yielded a 200-kilodalton membrane protein (Young et al., 1989). The CR2 receptor has been detected on three T-lymphoblastoid cell lines (Fingeroth et al., 1988) and on a fraction of normal human peripheral blood T lymphocytes (Fischer

et al., 1991), these findings suggest that the tropism of EBV for B lymphocytes may rely on factors other than receptor specificity.

Permissiveness of Virus Replication in Vitro

Cultures of EBV-infected lymphocytes vary in their permissiveness for viral replication, most cultures being nonpermissive, but replication does occur in a small fraction of cells in some cultures. The nonpermissiveness of EBV infection has made it difficult to study virus replication and also limits the amounts of purified virus available for studying the components of mature virus particles. Clones of infected lymphocytes that are more permissive of virus replication have been selected (Miller and Lipman, 1973) and have facilitated studies of the virus replication cycle and biochemical analyses. Two isolates of virus have been extensively studied, B95-8 and P3HR1. The B95-8 strain is produced by a cell line derived from a clone of marmoset lymphocytes that were infected with virus obtained from a culture of lymphocytes from a patient with infectious mononucleosis. The B95-8 viral DNA has been completely sequenced (Baer et al., 1984) and has been the prototype used for gene mapping. The P3HR1 cell line is a clone of the Jijoye Burkitt-tumor derived cell line (Hinuma et al., 1967). The P3HR1 cells are more permissive than the parent clone for virus replication and the virus produced by P3HR1 cells lacks the ability to growth-transform noninfected B lymphocytes (Miller et al., 1974).

Genome Structure

The linear double stranded EBV genome contains nonrandom single stranded breaks (Pritchett et al., 1975). When the genome is carried in the latent state it circularizes via joining of the terminal repeated DNA sequences at either end of the molecule (Dambaugh et al., 1980). The genome consists of five large regions of

unique DNA domains, U1-U5, which are separated by four regions of internal repeats, IR1-IR4, and flanked on both ends with tandem repeats (Cameron et al., 1987; Dambaugh and Kieff, 1982; Given et al., 1979). Latently infected cells usually contain more than one copy of the complete EBV genome, which can be integrated, but is most often found to exist as a covalently closed circular episome (Lindahl et al., 1976). Episomes are replicated once per cell cycle by DNA polymerase early in S phase (Adams, 1987; Hampar et al., 1974). Replication of the episomal DNA is proposed to occur from a circular form in a manner similar to that of SV40 DNA (Gussander and Adams, 1984).

At least two EBV types have been identified in human populations (Rowe et al., 1989; Sculley et al., 1988). The two strains have significantly divergent EBNA 2 sequences. These were designated EBV type A and B, but are more appropriately designated EBV-1 and EBV-2, so as to parallel the HSV-1 and HSV-2 nomenclature. EBV-1 and EBV-2 are considerably more closely related to each other than are HSV-1 and HSV-2. Analysis of hosts shedding both EBV types in the oropharynx revealed only type 1 in peripheral blood lymphocytes (Sixbey et al., 1989). Oral hairy leucoplakia lesions consistently contain EBV DNA of the type 2 (Raab-Traub and Sixbey, personal communication). The type 2 strain transforms B lymphocytes less efficiently than the B95-8 type 1 strain and B cell transformants of the type 2 are more difficult to maintain in culture (Rickinson et al., 1987).

Membrane Proteins

The membrane antigen complex was initially described by surface fluorescence of EBV-producing cells using human immune sera. The complex was further resolved by analysis of infected cell membranes into three major envelope glycoproteins of

300-350 kda, 200-220 kDa, and 85 kDa (Edson and Thorley-Lawson; Thorley-Lawson and Edson, 1979). Three additional membrane associated proteins, p105, gp78/55, and the product of the BDLF3 open reading frame, have also been studied. The p105 protein is not glycosylated and differs from the other membrane proteins in that its synthesis is not influenced by the viral DNA inhibitor phosphonoacetic acid (Balachandran et al., 1986). Glycoprotein gp78/55 is the product of the BILF2 open reading frame (Mackett et al., 1990). Antibodies to a bacterially expressed BDLF3 protein reacted with virus and with the plasma membrane of virus infected cells. Additional membrane proteins are likely to exist since there are many unassigned open reading frames which have characteristics of those encoding membrane proteins.

Glycoproteins gp300-350 and gp200-220 are present in large amounts in the virus envelope and have been extensively characterized. Glycoprotein gp350 and gp220 are encoded by the same open reading frame from which an intron is removed, without change in reading frame to produce gp220 (Beisel et al., 1985; Hummel et al., 1984). Monoclonal antibodies that recognize gp350/220 are capable of inhibiting virus binding (Nemerow et al., 1987). Binding of EBV to CR2 is mediated by attachment of gp350 (Nemerow et al., 1987; Tanner et al., 1987) and possibly also by attachment of gp220 (Wells et al., 1982). A common epitope in gp350 and gp220 has been identified as a primary region responsible for virus binding to B lymphocytes by attachment to CR2 (Nemerow et al., 1989).

Glycoprotein gp85 is also present in the envelope but in less abundant amounts than gp350/220. Glycoprotein gp85 has been recently mapped to the BXLF2 open reading frame in two independent studies (Heineman et al., 1988; Oba

and Hutt-Fletcher, 1988). Although no function has been conclusively ascribed to this molecule, antibodies to it can neutralize virus infectivity (Strnad et al., 1982), thus implying that it may play a role in the initiation of cell infection. The function of gp78/55 has not been determined; neither a monoclonal antibody nor a polyclonal sera to the recombinant molecule neutralized the ability of virus to transform cells.

Preliminary studies with recombinant vaccinia virus expressing the gene product from the BDLF3 open reading frame have immunoprecipitated a protein of 90kd using serum from a patient with chronic mononucleosis (L.C. Davenport and L.M. Hutt-Fletcher, personal communication).

Entry of Enveloped Viruses into Animal Cells

The earliest events in the virus replication cycle are attachment, penetration, and uncoating. The initial event, virus attachment to specific cell receptors, is a major determinant of cellular tropism and pathogenesis of viruses. Virus membrane proteins protruding from the virus envelope mediate virus attachment to host cells. These membrane proteins have other functions in addition to cell recognition and attachment, namely fusion, penetration, and possibly, direction of egress of the virus. Enveloped viruses enter cells by fusing with cellular membranes (Lonberg-Holm and Philipson, 1974; White et al,, 1983; White, 1990). Since fusion is an energetically unfavorable process, viruses utilize specific proteins to fuse with host cells and introduce their genetic material into the host cell (White, 1990). Two pathways of entry are commonly utilized and viral fusion reactions fall into two classes, low pH-dependent and pH-independent. Some viruses, such as Sendai (Scheid and Choppin, 1976), deposit their nucleocapsids directly into the cytoplasm by fusing with

the plasma membrane at physiologic pH. The alternative route, adsorptive endocytosis followed by vesicle membrane fusion, is utilized by Semliki Forest virus (SFV) (Helenius et al., 1980a; Marsh and Helenius, 1980), influenza A (Matlin et al., 1981; White et al., 1981; White et al., 1983), Sindbis (Boggs et al., 1989) and vesicular stomatitis virus (VSV) (Matlin et al., 1982; White et al., 1983). In most cases studied, fusion is induced by a specific viral membrane 'fusion protein'.

Adsorptive Endocytosis

Adsorptive endocytosis, also known as 'receptor-mediated' endocytosis, is a process by which macromolecules are taken into cells. This process is initiated by binding of a ligand to a cell surface receptor followed by invagination of the membrane forming a vesicle (Goldstein et al., 1979; Silverstein et al., 1977). Specialized regions of the plasma membrane have been morphologically identified as sites for adsorptive endocytosis of some viruses (Goldstein et al., 1979). These regions, the coated pits, are thought to concentrate receptors and receptor-ligand complexes at sites of internalization. The protein clathrin is a major component of the coated pits (Pearse, 1975) and is thought to participate in the early stages of endocytosis (Doxsey et al., 1987). The process of adsorptive endocytosis as a mechanism for virus entry has been documented for several viruses (White et al., 1981; White et al., 1983; White, 1990). Semliki Forest virus (SFV), a togavirus, has been widely studied since its isolation in 1944 (Smithbum and Haddow, 1944). It is one of the best characterized enveloped viruses due to its simple structure. The nucleocapsid envelope is a host derived lipid bilayer in which virus encoded glycoproteins are inserted. This virus gains entry into cells by accumulation in coated pits that are endocytosed. The endosomes become acidified, providing conditions

that trigger fusion of the virus envelope with the vesicle membrane (Helenius et al., 1980b; Kielian and Helenius, 1985; Marsh and Helenius, 1980; White and Helenius, 1980; White et al., 1980). The entry of vesicular stomatitis virus (VSV) has been reported to resemble that of SFV (Clague et al., 1990; Matlin et al., 1982). Influenza virus, an orthomyxovirus, is also taken into cells by adsorptive endocytosis followed by fusion of the viral membrane with the endosomal membrane (Matlin et al., 1981; White et al., 1981; Yoshimura and Ohnishi, 1984).

Fusion at the Plasma Membrane

Fusion directly at the plasma membrane is utilized by paramyxoviruses (Choppin and Compans, 1975a). The best studied member of this group is Sendai virus, whose glycoproteins have been extensively characterized. The entry of Sendai involves initial attachment of virions to the cell surface and subsequent fusion between the viral envelope and plasma membrane (Choppin and Scheid, 1980; White et al., 1983). It is well established that binding is mediated by the HN protein and fusion is initiated by the F protein, both of which are spike-like projections on the surface of the virus (Choppin and Scheid, 1980). Fusion activity has been shown to be critically temperature dependent, optimally occurring at 37°C, while fusion is insignificant at temperatures below 23°C (Hoekstra et al., 1984).

The major entry mechanism for human immuno deficiency virus (HIV), a T-lymphotropic retrovirus, is reported to be fusion with the plasma membrane at the cell surface (Maddon et al., 1988; McClure et al., 1988; Stein et al., 1987). Previous data from Maddon et al. (1986), proposed that HIV entry into T lymphoblastoid cells occurred after endocytosis because the virus receptor, CD4, was internalized. The key findings of Stein et al. showed that the entry of HIV was not low-pH-dependent,

and although they found no evidence of an endocytic entry pathway, they did not rule out the possibility that virus could enter by both pathways in a pH-independent manner. Analysis of cells expressing a mutant form of CD4 that had impaired ability to undergo endocytosis revealed that HIV infection did not require endocytosis of its receptor, CD4 (Maddon et al., 1988).

For EBV, studies utilizing electron microscopy and immunoelectron microscopy have reported direct fusion at the plasma membrane of EBV with the lymphoblastoid cell line, Raji (Nemerow and Cooper, 1984a; Seigneurin et al., 1977). Virus nucleocapsids were found in the cytoplasm directly beneath the cellular plasma membrane, while virus was never found to be bound to the clathrin-coated areas of the plasma membrane, nor observed in endocytic vesicles. The same studies using normal B lymphocytes revealed transfer of membrane bound virus into vesicles. These vesicles were distinct in size and appearance from clathrin-coated vesicles. After 30 minutes at 37°C very few virus particles remained in the vesicles.

Membrane Fusion Proteins

A virus envelope has a relatively simple protein composition that has three main functions: facilitation of assembly and egress of virus particles, protection of the genome during the extracellular transport of virus, and delivery of nucleocapsids into host cells. The following viruses have proteins well characterized for ability to mediate viral and cell fusion: Sendai, Semliki Forest, influenza, and vesicular stomatitis virus. Fusion proteins identified to date are glycoproteins which span the bilayer and have the bulk of their mass exposed externally. The transmembrane anchor region of the glycoprotein is frequently composed of hydrophobic residues that favor alpha helix formation.

The envelope of Sendai virus, a paramyxovirus, has two proteins. The hemagglutinin-neuramidase (HN) protein is responsible for attachment of the virus to cell surface sialic acid residues. The fusion (F) protein initiates fusion at the plasma membrane allowing virus penetration, virus-induced cell fusion and hemolysis (Hsu et al., 1981; Scheid and Choppin, 1974; Scheid and Choppin, 1976). The F protein consists of two sulfhydryl-linked glycopeptides (F_1 and F_2) , resulting from proteolytic cleavage of an inactive precursor (F_0) by a host cell enzyme (Hsu et al., 1982). Viruses produced by cells that lack a suitable protease for F protein activation are noninfectious (Hsu et al, 1982). F_2 corresponds to the N-terminus of F_0, and the protein is anchored in the bilayer through F_1. The N-terminus of F_1, resulting after cleavage of F_0 has been found to be unusually hydrophobic (Gething et al., 1978) and it was suggested that the hydrophobic terminal peptide might play a role in fusion. Support for this role has been provided by experiments with synthetic peptides corresponding to the hydrophobic amino-terminus of F_1 showing that such molecules inhibit virus fusion (Richardson et al., 1980). The amino acid sequence in this region is highly conserved among paramyxoviruses (Scheid et al., 1978).

Orthomyxoviruses also have two types of spike glycoproteins which have neuraminidase, hemagglutination, and fusion activities. One of the glycoproteins is a neuraminidase (NA) and the other, the hemagglutinin (HA), has the capability to bind to cell surface sialic acid residues and to catalyze fusion (Choppin and Compans, 1975b; White et al., 1982). Unlike paramyxoviruses, orthomyxoviruses are endocytosed and fuse with the endocytic vesicle. The HA consists of two disulphide linked glycopeptide chains, HA_1 and HA_2, resulting from proteolytic cleavage of a precursor glycoprotein HA_0. The cleavage is irrelevant to adsorption, but is a

prerequisite for infectivity (Lazarowitz and Choppin, 1975; White et al., 1983). The cleavage generates a new N-terminus on HA_2 which is hydrophobic and highly conserved in different influenza strains and has partial homology with the N-terminus of F_1. Synthetic peptides analogous to the N-terminus sequence of HA_2 inhibit infectivity by influenza viruses (Gething et al., 1986; Richardson et al., 1980). The HA molecule in its neutral form is a trimer and the hydrophobic fusion peptide in each monomer is unexposed until the low pH of the endocytic vesicle causes partial dissociation of the HA trimer, thus exposing the fusion peptide which can insert into the target bilayer (Doms et al., 1985; Schlegel et al., 1982) and initiate endosomal membrane fusion. Collective research findings suggest that the pH induced conformation does not involve any changes in secondary structure and that the stem region of the spike remains trimeric. However, elements of the spike change their relative positions with the globular heads dissociating from one another by bending about a hinge region. This movement of the three proteins composing the spike is thought to release the terminal fusion peptide from the molecular interior (Doms et al., 1990; Doms and Helenius, 1988; Harter et al., 1989; Ruigrok et al., 1988; Stegmann et al., 1987, 1989; Wharton, 1987; Wharton et al., 1988; White et al., 1983; White and Wilson, 1987; Wiley and Skehel, 1987). The HA is the only membrane fusion protein for which a crystal structure is known (White, 1990).

The envelope spike of Semliki Forest virus (SFV), a togavirus, consists of a complex of three glycopeptides, E1, E2, and E3. E1 and E2 are transmembrane glycoproteins; E3 is noncovalently associated with E2 and is external to the bilayer. This virus does not fuse with the plasma membrane at physiologic pH (Helenius et al., 1980a). Virions are endocytosed and a fall in pH within the endocytic vesicle activates

membrane fusion (Marsh et al., 1983a). Lysosomotropic agents, which elevate endosomal pH, inhibit SFV penetration (Helenius et al., 1982). Semliki Forest virus can fuse directly with the plasma membrane in vitro at low pH (White et al., 1980). The SFV spike glycoproteins have been shown to be fusogenic in the absence of other virus components (Marsh et al., 1983b). As far as the role of the glycopeptides are concerned, it has been suggested that the peptide E1 may be directly involved in the fusion activity (Kielian and Helenius, 1985). Both SFV and Sindbis, another togavirus, have E1 proteins containing a hydrophobic peptide segment located close to the N-terminus, and this segment has an external position in the virus membrane (Garoff et al., 1980; White et al., 1983). Since E1 and E2 occur as a complex, E2 may also participate in the fusion reaction. The role of E3 is not clear, it is a small peripheral glycopeptide and there is no homologue in Sindbis virus (Welch and Sefton, 1979).

Vesicular stomatitis virus (VSV), a rhabdovirus, has only one type of envelope glycoprotein, designated the G-protein. The G-protein has a hydrophobic region near the C-terminus forming the intramembranous domain. A small hydrophilic sequence at the C-terminus is in contact with the cytoplasm. The larger N-terminal domain, containing the oligosaccharide chains, is exposed to the exterior of the cell (Rose et al., 1980; Rose and Gallione, 1981). The G-protein has been cloned and sequenced (Rose and Gallione, 1981). Eukaryotic cells expressing the cloned G-protein gene fuse, at low but not at neutral pH, indicating that this protein is both necessary and sufficient for fusion activity (Reidel et al., 1984). In addition, at low pH, the G-protein spikes reversibly aggregate at the ends of virus particles (Brown et al., 1988); this observation may be potentially relevant to determining the mechanism of fusion for

this virus. The fusion activity has been shown to occur at the plasma membrane if cells with VSV attached to their surfaces are placed in a low pH medium (Blumenthal et al., 1987; Matlin et al., 1982).

Herpesviruses are considerably more complex. The best studied, herpes simplex virus (HSV), has an envelope that contains at least nine glycoproteins, five of them have been characterized and sequenced (Bzik et al., 1984; Frink et al., 1983; Gompels and Minson, 1986; McGeoch et al., 1985; Pellet et al., 1985; Watson et al., 1982). Studies indicate that the receptor molecules recognized in one of the initial binding events are heparan sulfate proteoglycans (WuDunn and Spear, 1989). Recently, it was determined that glycoprotein gC is principally responsible for virus adsorption to cells (Herold et al., 1991). Glycoprotein gC bound heparin and virions devoid of gC exhibited significant impairment in adsorption and penetration. Three of the glycoproteins, namely gB, gD, and gH, induce antibodies capable of neutralizing HSV infectivity in the absence of complement and have been implicated in virus penetration (Fuller and Spear, 1987; Gompels and Minson, 1986, Sarmiento et al., 1979). Evidence implicating gB in penetration comes from studies of temperature sensitive HSV-1 mutants that fail to process precursor gB molecules to mature forms at nonpermissive temperature. The virions produced are noninfectious but can bind to cells and the block to their infectivity can be overcome by treating virus-cell complexes with the membrane fusing agent polyethylene glycol (Little et al., 1981; Sarmiento et al., 1979). Neutralizing anti-gD monoclonal antibodies have been shown to block HSV infection by preventing virus-cell fusion at the plasma membrane (Fuller and Spear, 1987) and antibodies to this glycoprotein also block HSV-induced cell-cell fusion, a process which may be analogous to the virus-cell fusion required for entry

(Noble et al., 1983). Virus lacking gB (Cai et al., 1988) or gD (Johnson and Ligas, 1988) attaches but does not penetrate. The glycoprotein gH is present in the viral envelope at concentrations at least 10-fold lower that gD (Richman et al., 1986). Despite this fact, antibodies against gH have neutralizing activity comparable to that of antibodies against gD (Minson et al., 1986). A monoclonal antibody to gH has also been shown to exhibit anti-fusion activity (ibid). Thus three glycoproteins, gB, gD, and gH, are likely either to induce or influence the fusion process which occurs in a pH-independent manner at the surface of the cell. There is no evidence to suggest that they act as a single functional heteropolymer. Homodimers of gB extracted from virions or infected cells are not associated with other glycoproteins (Claesson-Welsh and Spear, 1986), and gB and gD have been shown to form morphologically distinct structures in the virion envelope (Stannard et al., 1987).

Entry of Epstein-Barr Virus

Infection of B lymphocytes and epithelial cells with EBV is initiated by attachment of virus to a 145-kilodalton cell membrane glycoprotein, CR2, which also serves as the receptor for the C3d fragment of the complement cascade (Cooper et al., 1990; Fingeroth et al., 1984; Nemerow et al., 1985b; Sixbey et al., 1987). Expression of the CR2 molecule on both cell types is linked to cell differentiation. CR2 expression on human B lymphocytes is lost at the plasma cell stage of differentiation (Tedder et al., 1984). Immunofluorescent studies have demonstrated expression of CR2 on epithelia in a differentiation-linked manner as it is on B lymphocytes (Sixbey et al., 1987; Young et al., 1986, 1989). Binding of EBV to CR2 is mediated by attachment of at least one virus membrane glycoprotein, gp350

(Nemerow et al., 1987; Tanner et al., 1987), and possibly also by attachment of gp220 (Wells et al., 1982).

Penetration of virus has been studied in normal B cells and lymphoblastoid cell lines. Virus fuses with the membrane of the lymphoblastoid cell line Raji at the cell surface and CR2 is not internalized (Nemerow and Cooper, 1984a; Tedder et al., 1986). In normal B cells, both receptor and virus are endocytosed into thin-walled nonclathrin coated vesicles before fusion occurs (ibid).

The virus envelope protein mediating the fusion event has not been conclusively identified. The EBV envelope glycoprotein, gp85, which has been recently mapped to the BXLF2 open reading frame of EBV DNA does, however have, characteristics of a fusion protein (Oba and Hutt-Fletcher, 1988; Heineman et al., 1988). Computer assisted analysis of the sequence indicates that it is overall a hydrophobic molecule with a potential N-terminal signal sequence and a C-terminal anchor sequence. The sequence also includes a stretch of 16 extremely apolar amino acids that could be a fusion sequence (Oba and Hutt-Fletcher, 1988). The gp85 glycoprotein has homology with the herpes simplex virus glycoprotein gH, and the varicella-zoster virus gpIII, which are involved in cell to cell fusion.

Measuring Fusion

The common procedures used to examine fusion of biological membranes, such as microscopic or cytochemical techniques, are frequently difficult to quantitate and have low sensitivity; extensive fusion activity may be required before it can be detected. The use of radioisotopes to measure fusion does not permit continuous monitoring of the fusion process and it is necessary to separate fused and nonfused

membranes in order to quantitate fusion events. Electron spin labels have been used extensively with virus systems (Maeda et al., 1975, 1981, Lyles and Landesberger, 1979) but the extent of fusion is difficult to quantitate and continuous monitoring of the fusion event is technically challenging. Assays utilizing fluorescent probes are much faster and easier to perform than assays using electron spin probes and easily permit continuous monitoring of the fusion events. The assay presented and utilized throughout this work relies upon the relief of fluorescence self-quenching of the fluorophore octadecyl rhodamine B chloride.

Quenching of fluorescence intensity can occur by a variety of mechanisms. These include collisional processes with specific quenching molecules, excitation transfer to nonfluorescent species, and complex formation or aggregation that forms nonfluorescent species, also known as concentration quenching. Quenching of fluorescence by added substances or by impurities can occur by a collisional process. Molecular oxygen is one of the most widely encountered quenchers. This is because O_2 is a triplet species in its ground electronic state and is able to transfer unpaired electrons to the fluorescent species which is in the singlet state. The fluorescence quenching of octadecyl rhodamine B chloride (R_{18}) is due to complex formation and is dependent upon the concentration of the fluorophore in the lipid-containing membrane. The self-quenching is concentration dependent because of the formation of excimers (excited dimers) when interactions of the excited-state species occurs. Most excited fluorophores emit fluorescence from a singlet state. The formation of dimers results in quenching since the doublet species is not fluorescent (Tinoco et al., 1985). The efficiency of self-quenching is directly proportional to the ratio of R_{18} to total lipid. When the fluorophore is incorporated into a lipid bilayer at concentration

up to 9 mol% with respect to total lipid, the efficiency of the self-quenching is proportional to its surface density (Hoekstra et al., 1984). When fusion of a labeled membrane with a nonlabeled membrane occurs, there is a decrease in the surface density of the fluorophore and this results in a proportional relief of the self-quenching.

Purpose of this Work

The overall objective of this dissertation is to understand how Epstein-Barr virus enters its two target cells, the B lymphocyte and the epithelial cell. A greater understanding of the conditions for successful virus penetration into both epithelial cells and lymphocytes, as well as the viral components necessary to mediate these events, will help to understand the unique tropism of EBV for B lymphocytes and epithelial cells. This work presents experiments undertaken to develop an assay for measuring fusion of EBV with cell membranes and application of the assay to follow fusion with lymphoblastoid cells, B lymphocytes, and epithelial cells.

CHAPTER 2
ESTABLISHMENT OF AN ASSAY TO MEASURE VIRUS FUSION

Introduction

Membrane fusion is an effective process for delivering membrane-bound contents from one cellular compartment to another. Viruses take advantage of this important process and utilize membrane fusion for entry into cells. The mechanism of fusion has become one of the most intriguing questions in cell biology, and viruses provide a natural experimental system for studying the fusion process. Fusion of two lipid bilayers is an energetically unfavorable process, and the fact that viruses, which have relatively simple membranes, use this process for entry into cells makes them a very interesting model for studying membrane fusion.

Studies of membrane fusion have, in the past, been largely morphological and descriptive due to lack of techniques for measuring and analyzing the fusion process in isolation. Many assays used involved radioisotopes (Haywood and Boyer, 1982; White et al., 1983) and electron spin probes (Maeda et al., 1975, 1981; Lyles and Landesberger, 1979) or involved use of indirect techniques such as hemolysis and infectivity (White et al., 1983). Of all these techniques only electron spin resonance permits the continuous monitoring of the fusion process, which is desirable in a fusion assay. More recently, the assay described in this work has been widely adopted for measurement of membrane fusion in isolation from other events in the virus life cycle that precede or follow it. This method not only allows for continuous monitoring of

fusion between membranes but also provides an opportunity to analyze the kinetics of fusion between membranes, which can be useful for comparing the kinetics of virus fusion with different cell types.

Materials and Methods

Lymphoblastoid Cell Lines

Cell lines were grown at 37°C and diluted at least biweekly in RPMI 1640 (Sigma Chemical Co., St. Louis, Missouri) supplemented with heat-inactivated fetal calf serum (5-10%, depending on cell type), 100 IU of penicillin and 100ug of streptomycin per milliliter. The cell lines used include four human EBV genome-positive B lymphoblastoid cell lines, Raji (Pulvertaft, 1964), Daudi (Klein et al., 1968), P3HR1-Cl13 and P3HR1-Cl5 (Heston et al., 1982). Raji is a latently infected virus nonproducing cell line expressing CR2. Daudi is a genome positive nonproducing cell line that currently, in our laboratory, does not express CR2. P3HR1-Cl13 is a superinducible virus producing cell line and P3HR1-Cl5 is a genome positive cell line derived from the same parent line as P3HR1-Cl13, but currently in our laboratory does not produce virus. Also used were MCUV5, an EBV producing marmoset cell line and Molt 4 (Minowda et al., 1972), an EBV genome negative human T cell line that expresses CR2, but cannot internalize virus (Menezes et al., 1977).

Virus Production and Radiolabeling

A small percentage of the P3HR-Cl13 cells spontaneously produce low levels of virus, but this amount can be increased after induction with 30ng of 12-O-tetradecanoyl phorbol-13-acetate (TPA) per milliliter (Sigma). The virus obtained from

the MCUV5 cell line will transform fresh human B cells and induce them to secrete immunoglobulin (Gerber and Lucas, 1972), whereas the P3HR1-Cl13 virus is a non-transforming lytic strain of virus. Virus was obtained from producer cells by harvesting the virus from culture supernatant 7 days after induction with TPA. Cell culture supernatant was cleared of cells by centrifugation at 4,000 x g for 10 minutes. Bacitracin (Sigma) was added to the clarified supernatant (100 ug/ml) to reduce virus aggregation, and the virus was pelleted by centrifugation at 20,000 x g for 90 minutes. The virus pellets were resuspended in 1/250 original volume of medium containing 100ug per ml bacitracin, reclarified of cell debris by centrifugation three to four times at 400 x g, and filtered through a .45um-pore filter (Acrodisc, Gelman Sciences, Inc., Ann Arbor, Michigan).

P3HR1-Cl13 virus was intrinsically labeled with (^{3}H) thymidine (^{3}HTdR; Amersham Corp., Arlington Heights, Illinois) by feeding cells with medium containing 100uM hypoxanthine and 0.4uM aminopterin (Sigma), inducing them with TPA when they reached confluency (day 0) in the presence of 2 uCi of 3HTdR (specific activity 5 Ci/mmol) per ml, adding an additional 2uCi of 3HTdR (specific activity 52 Ci/mmol) on day 3, and harvesting the virus on day 7 in the same manner as described above. All virus stocks were stored at -70°C.

Monoclonal Antibodies

Monoclonal antibodies were purified from hybridoma culture supernatants by chromatography on protein A-Sepharose (Genzyme, Boston, Massachusetts). The antibody 72A1 (Hoffman et al., 1980) is an IgG1 antibody that recognizes the viral glycoprotein gp350/220. Two monoclonal antibodies that react with CR2 were used,

OKB7 (Rao et al., 1985), which blocks virus binding, and HB5, which does not block the virus binding site of CR2 (Nemerow et al., 1985a).

Virus Binding Assay

The ability of intrinsically radiolabeled virus to bind specifically to CR2 was determined by use of receptor positive and negative cells that had been briefly fixed with ice-cold 0.1% paraformaldehyde. Virus was incubated with 2×10^6 fixed cells for 60 minutes at 4°C, cells were washed five times to remove unbound virus and the acid-precipitable radioactivity that remained attached to cells was counted. The ability of antibody to interfere with virus binding was determined by preincubation of virus and antibody for 1 hour at room temperature.

Isolation of B-cell Enriched Leukocytes

Heparinized human peripheral blood was separated by flotation on Lymphocyte Separation Medium (LSM; Litton Bionetics, Charleston, South Carolina). T cells were depleted from the leukocyte fraction by a double cycle of rosetting with 2-aminoethyl isothiouronium bromide-treated sheep erythrocytes (Pellegrino et al., 1975) and centrifugation over 60% isotonic Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey). The nonrosetting cells remain at the RPMI-Percoll interface and are collected and washed free from remaining Percoll.

Human tonsil tissue was teased apart with forceps and rinsed with RPMI to collect single cells. Cells were washed and resuspended in RPMI and separated on LSM. T cells were depleted by rosetting as stated previously.

The T-depleted leukocytes were also depleted of monocytes in some experiments by one of two methods. Monocytes were depleted by adherence to plastic petri dishes in 10% RPMI for 1 hour at 37°C and the nonadherent cells were

collected. Alternatively, cells were incubated with iron filings in a 15 ml polypropylene tube at 37°C on a rotator for 1 hour and the iron containing cells were removed with a magnet. The remaining cells were layered on lymphocyte separation medium for additional removal of iron containing cells. The extent of monocyte depletion was determined by cell counts and nonspecific esterase staining of cells prior to and after depletion.

Nonspecific Esterase Stain

Nonspecific esterase is contained in the granules of monocytes and was stained with a solution of Sorensen's buffer, hexazotised pararosaniline and alpha-naphthyl butyrate (Li et al., 1973). Sorensen's buffer consists of 0.2M Na_2HPO_4 and 0.2M NaH_2PO_4 at pH 6.3. Hexazotised pararosaniline was prepared by mixing equal volumes of pararosaniline HCL and 4% sodium nitrite. One gram of alpha-naphthyl butyrate was dissolved in 50ml of dimethyl formamide and stored at -20°C, protected from the light. To prepare the primary stain, 0.25ml of hexazotised pararosaniline and 3.0ml of alpha-napthyl butyrate were added to 44.5ml of Sorensen's buffer and the solution was filtered through a Whatman #1 filter and 5 X 10^6 fixed cells were stained for 30 to 45 minutes at 37°C. The slides were rinsed with deionized water and counterstained in methyl green for 15 seconds. The slides were rinsed again in deionized water and air dried. Monocytes were identified by the brown coloration of their cytoplasm.

Initiation of an Immortalized B Cell Line

T-depleted leukocytes were isolated from peripheral blood as previously described. The cells were plated in a 24-well tissue culture plate at a concentration of 2 X 10^6 per milliliter and 100ul of virus were added to each well using a twofold

dilution series starting at 1:10. Clonal outgrowths were selected and propagated in RPMI 1640 with 10% fetal calf serum. A cell line initiated in this manner, designated BAT, was utilized for comparing virus fusion with immortalized B cells, freshly isolated human B cells and lymphoblastoid cell lines derived from tumor tissues that had been in culture for many years.

Virus Titration and Neutralization

Infectivity of EBV was measured in terms of its ability to induce human peripheral B lymphocytes to secrete immunoglobulin in culture (Kircher et al., 1979). T-depleted leukocytes were incubated with or without virus at 37°C in 96-well round-bottomed tissue culture plates at concentrations of 10^5 cells per well in 100ul of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100IU of penicillin per milliliter, and 100ug of streptomycin per milliliter. After 6 days in culture, 100ul of medium were added to each well. On day 12, the culture supernatants were collected and the immunoglobulin concentrations were measured. The ability of antibody to neutralize infectivity was determined by preincubating virus for 1 hour at room temperature with an equal volume of normal rabbit antibody at 100ug per ml, or with mixtures of rabbit antibody and test antibody adjusted so that the total amount of immunoglobulin remained constant at 100ug per ml. All antibodies were heated for 30 minutes at 56°C to inactivate complement prior to incubation with virus.

Incorporation of Octadecyl Rhodamine B Chloride (R_{18}) into Virus Membranes

Octadecyl rhodamine B chloride (R_{18}), is a fluorescent amphiphile that can be readily inserted into biological membranes (Figure 2-1). A stock solution of 13 nmoles/ul of R_{18} (Molecular Probes, Inc., Junction City, Oregon) was prepared in chloroform/methanol (1:1) and stored at -20°C. The probe was incorporated into virus

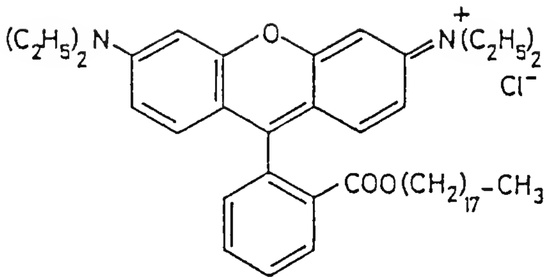


Figure 2-1. Structural formula of octadecyl rhodamine B chloride (R_{18}).

membranes by modification of the method of Hoekstra et al. (1984). Three microliters of stock probe were dried under nitrogen and solubilized in 39ul absolute ethanol and 15ul of this solution, containing 15nmole R_{18}, were added to 250ul of concentrated virus under vortexing. For mock-labeled virus the same volume of absolute ethanol was added to the virus as used in the labeling procedure. Probe and virus were incubated at room temperature in the dark for 1 hour. Virus and nonincorporated R_{18} were separated by chromatography on Sephadex G-75 (Sigma Chemical Co., St. Louis, Missouri). Labeled virus was aliquoted and stored at -70°C.

Fluorescence Dequenching Assay

An Aminco-Bowman spectrofluorometer (SLM Amino Bowman Instrument Co., Urbana, Illinois), equipped with a chart recorder was used for continuous monitoring of fluorescence. The cuvette chamber was equipped with a magnetic stirrer and held in a temperature controlled circulating water bath. For fluorescence measurements, the instrument was calibrated such that any residual fluorescence of membranes at time zero was set at the zero level. At the end of the assay, Triton X-100 (Sigma) (1.0% v/v, final concentration) was added to allow measurement of the maximum obtainable fluorescence for the virus bound upon infinite dilution of the fluorophore.

R_{18}-labeled virus (volume not exceeding 100ul) were added to pellets of 2 x 10^6 cells and incubated for 1 hour at 4°C on ice and in the dark. Cells were washed four times with ice cold Dulbecco's saline and transferred to the microcuvette of the spectrofluorometer. The principle of the assay relies upon the self-quenching properties of R_{18} when inserted into the virus membrane. When the two fusing membranes come into molecular contact their lipid components must mix and this mixing dilutes the R_{18} allowing relief of the self-quenching. The relief of self-quenching

of the R_{18} was continuously monitored at excitation wavelength of 560nm and emission wavelength of 585nm and documented with a chart recorder.

Immunoglobulin Assay

Immunoglobulin in culture supernatants was measured by a double sandwich micro-enzyme-linked immunosorbent assay (Voller et al., 1976) using rabbit anti-human immunoglobulin as the immobilized antigen. Antibody in the culture supernatants was allowed to bind to the immobilized antigen followed by horseradish peroxidase-conjugated rabbit anti-human Ig (Cooper Biomedical Inc., Malvern, Pennsylvania). The substrate, hydrogen peroxide with 5-amino salicylic acid was degraded by the enzyme and the colorimetric change was measured at 492 nm.

Results

Fluorescence Properties of R_{18} Labeled Virus

Figure 2-2 shows the excitation and emission spectra of octadecyl rhodamine B chloride (R_{18}) incorporated into virus membranes when relieved of self-quenching with Triton X-100 (1% v/v final concentration). The excitation spectrum exhibits a maximum peak at 560 nm. The peak emission wavelength displayed a maximum at 585 nm. The emission wavelength has been shown to be dependent upon the environment of the probe (Hoekstra et al., 1984) with variance between 569 and 590 in different solvents. Figure 2-3 demonstrates the stability of the quenching of the fluorophore within the virus membrane and subsequent relief of quenching upon addition of Triton X-100 (1% v/v).

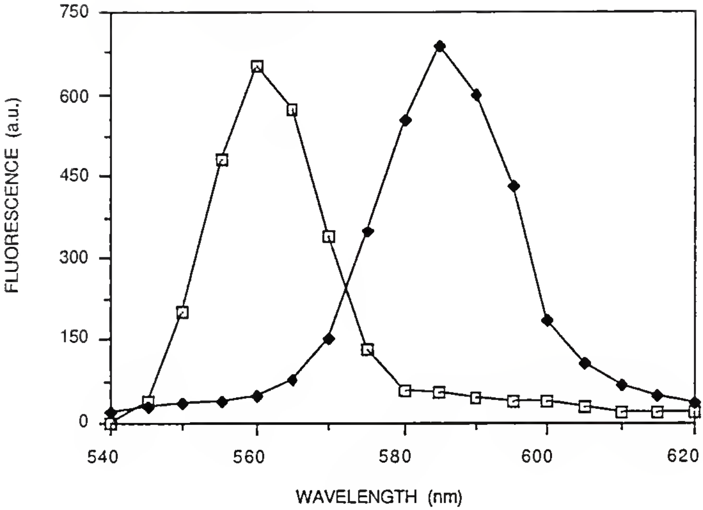


Figure 2-2. Excitation (—□—) and emission (—◆—) spectra of R_{18}-containing virions relieved of self-quenching with Triton X-100 (infinite dilution). Relative fluorescence expressed in arbitrary units (a.u.).

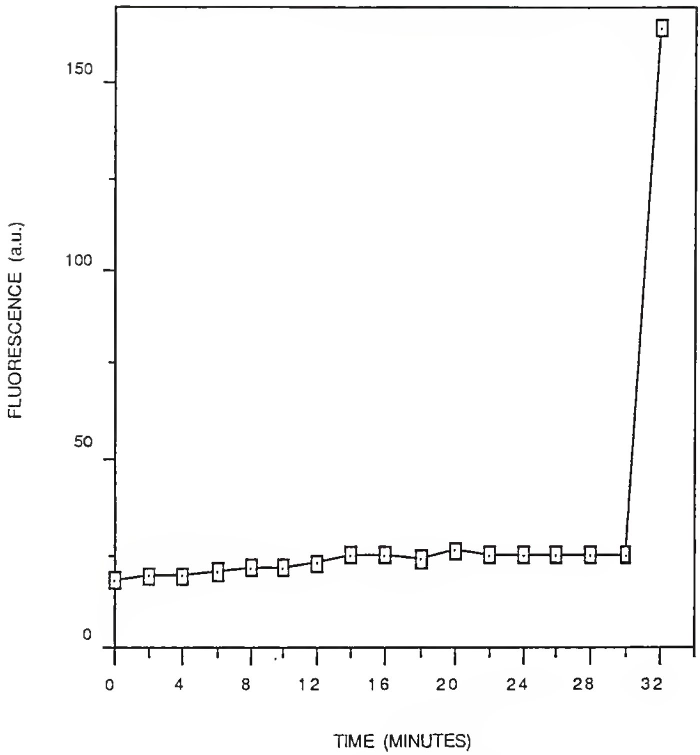


Figure 2-3. Stability of self-quenching of R_{18}-labeled virions maintained at 37°C and relief of self-quenching upon addition of Triton X-100 (infinite dilution) after 30 minutes. Relative fluorescence expressed in arbitrary units (a.u.).

Effect of R_{18} on the Attachment of Virus

When adapting the fluorescence assay of Hoekstra and colleagues for use with EBV, our first question was whether labeling of the virus with the fluorescent molecule would qualitatively or quantitatively affect virus binding. To answer this question we labeled virus metabolically with ^{3}HTdR, divided the virus into three aliquots, left one untreated, labeled one with an ethanolic solution of R_{18} and mock-labeled the third aliquot with ethanol alone. The labeled and mock-labeled preparations of virus were chromatographed on Sephadex G-75. A virus binding assay was done and the amount of radioactivity bound to receptor positive and negative cells was measured. Approximately half the bindable virus was lost during the labeling and mock-labeling procedures. However, if the amount of radioactivity bound was expressed as a percentage of the amount added, it could be seen that the labeling had no effect on the ability of the virus to bind to receptor positive cells (Table 2-1). There was no increase in nonspecific binding to receptor negative P3HR1-Cl5 cells.

The specificity of binding was further confirmed by showing that preincubation of virus with antibody 72A1 inhibited its ability to bind to receptor positive cells (Table 2-2). Two additional antibodies that have anti-CR2 activities were used in this experiment. Preincubation of cells with one, OKB7, which normally blocks virus binding (Nemerow et al., 1985a), inhibited labeled virus binding; preincubation of cells with HB5, a monoclonal antibody to CR2 that does not block the virus binding sites, appropriately failed to inhibit binding of labeled virus (Table 2-2).

Effect of R_{18} on Infectivity of Virus

Although the incorporation of R_{18} into the virus membrane did not alter the binding properties of the virus, it remained possible that the probe interfered with a

Table 2-1. Effect of labeling with R_{18} on the ability of [^{3}H] EBV to bind to receptor positive and negative cells.

Virus treatment	Virus dilution	Total acid precipitable counts bound to:		% acid precipitable counts bound to:[a]	
		Raji[b]	Cl5[c]	Raji	Cl5
None	neat	13284	543	28.9	1.2
	1/2	6346	-[d]	27.6	-
	1/4	2704	217	23.5	1.8
	1/8	1408	-	24.5	-
Mock-labeled	neat	5816	113	29.4	0.6
	1/2	2663	-	26.8	-
	1/4	1366	38	27.5	0.7
	1/8	758	-	30.6	-
R_{18}-labeled	neat	6962	248	25.5	1.0
	1/2	3707	-	27.1	-
	1/4	1831	104	26.8	1.5
	1/8	1016	-	29.7	-

[a]radioactivity bound/radioactivity added X 100
[b]receptor positive cells
[c]receptor negative cells
[d]not done

Table 2-2. Effect of monoclonal anti-EBV and anti-CR2 antibodies on the ability of R_{18}-labeled [^{3}H] EBV to bind to receptor positive cells.

Antibody (ug)	R_{18}-labeled virus		Mock-labeled virus	
	Total cpm bound	% cpm bound[b]	Total cpm bound	% cpm bound
none	1904	23.5	2789	23.9
72A1 (10)[a]	123	1.5	231	1.9
OKB7 (5)	198	2.4	180	1.5
HB5 (5)	1390	17.1	2308	19.8

[a]amount of antibody used, expressed in micrograms
[b]radioactivity bound/radioactivity added X 100

subsequent event in virus replication. Since the labeled virus was being used for studying events post binding it was necessary to examine the infectivity of labeled virus. A comparison was made of the ability of labeled and mock-labeled MCUV5 virus to induce immunoglobulin synthesis in cultures of T cell-depleted peripheral leukocytes (Table 2-3). There was no indication that incorporation of probe into virus had any detrimental effect on its biologic activity.

Changes in Fluorescence after Interaction of R^{18}-Labeled Virus with Lymphoblastoid cells

Figure 2-4 demonstrates the changes in fluorescence emission observed as virus bound to Raji cells at 4°C was warmed in the cuvette of the spectrofluorometer. The fluorescence increased gradually over approximately 28-32 minutes, after which time a plateau was reached. At this time, Triton X-100 (1% v/v) was added to relieve any residual self-quenching of the fluorophore and thus providing a rough approximation of the percentage of bound virus that fused. In Figure 2-4, 56% of the maximal fluorescence was reached, this value proved reproducible for this particular batch of labeled virus. The maximal value obtained with any batch of virus was 75%. Parallel analysis of the receptor negative Daudi cell line confirmed that R_{18}-labeled virus failed to bind to these cells (Figure 2-4). This result also showed that there was no significant diffusion of residual free or incorporated probe from the virus preparation into cell membranes during the 1 hour incubation at 4°C.

Further data indicating that relief of self-quenching was measuring a membrane fusion event and not simple diffusion of probe from closely approximated membranes were obtained using fixed Raji cells and the Molt 4 cell line (Figure 2-5). The increase in fluorescence emission measured when virus was bound to Raji cells at 4°C and then warmed to 37°C was almost completely eliminated if the cells were fixed with

Table 2-3. Effect of labeling with R_{18} on the ability of MCUV5 virus to induce immunoglobulin synthesis by fresh T-depleted human leukocytes.

Virus dilution	Immunoglobulin conc. ng/ml with:	
	R_{18}-labeled virus	Mock-labeled virus
1/5	24,754	22,366
1/10	45,720	23,836
1/20	38,609	23,639
1/40	39,902	27,404
1/80	42,326	30,105
1/160	33,921	21,093
1/320	18,168	15,999
1/640	3,970	not done
none	1,138	

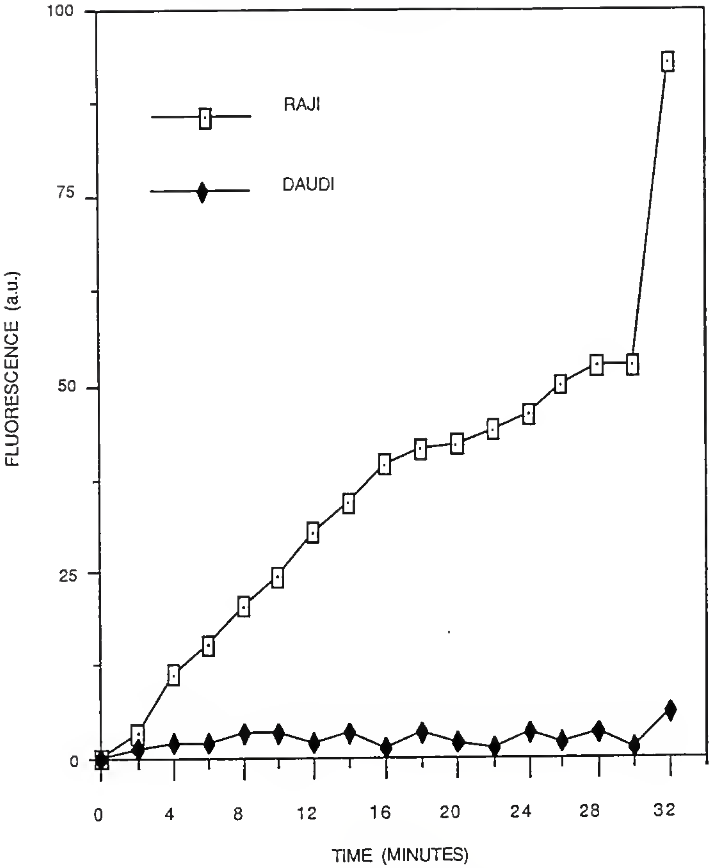


Figure 2-4. Relief of self-quenching of R_{18}-labeled virus bound to receptor positive Raji cells and receptor negative Daudi cells. At 32 minutes Triton X-100 was added to measure maximum relief of self-quenching of bound probe (infinite dilution). Relative fluorescence expressed in arbitrary units (a.u.).

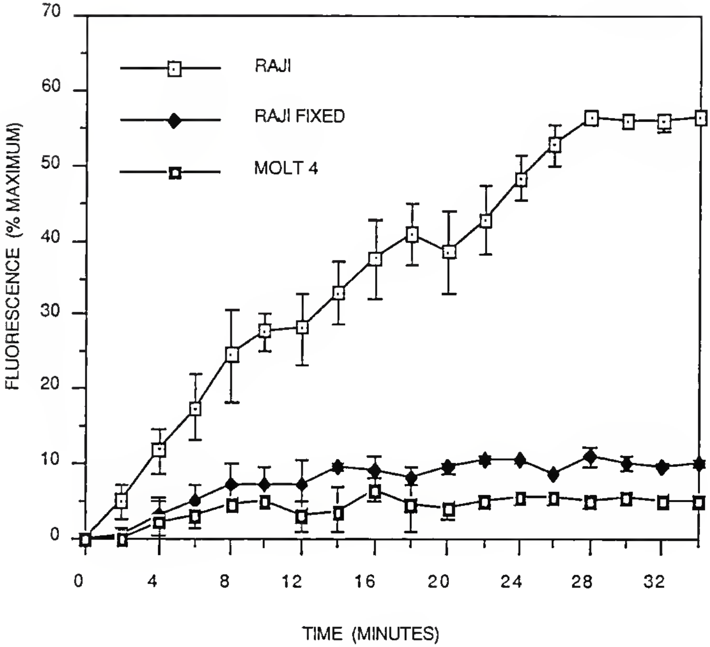


Figure 2-5. Comparison of relief of self-quenching of R_{18}-labeled P3HR1-Cl13 virus bound to Raji cells, fixed Raji cells, or Molt 4 cells. Increase in fluorescence is expressed as a percent of the maximum release obtained with each cell line after addition of Triton X-100 (infinite dilution). The average maximum fluorescence for each cell line was: Raji, 100a.u.; fixed Raji, 97 a.u.; Molt 4, 75 a.u.. Vertical lines indicate the standard deviation of the mean of experiments with the same batch of labeled virus.

paraformaldehyde prior to binding to virus. When Molt 4 cells were substituted in the assay for Raji, there was no significant relief of self-quenching of the bound probe, which is compatible with the reported inability of virus to fuse with Molt 4 cell membranes (Menezes, 1977). The fluorescence maxima obtained after addition of Triton was slightly less for Molt 4 cells than Raji, which is in agreement with published observations showing that Molt 4 cells express fewer receptors than Raji (Stoco et al., 1988).

Changes in Fluorescence after Interaction of R_{18}-Labeled Virus with Normal B Cells

Two independent studies have demonstrated that although EBV fuses with the plasmalemma of lymphoblastoid cells, it is endocytosed into normal B cells before any fusion of virus and cell membranes occurs (Nemerow and Cooper, 1984a; Tedder et al., 1986). However, if fusion was occurring within an endocytic vesicle, it seemed possible that the event might still be detectable with the R_{18}-labeled virus.

Experiments were done initially with B cells isolated from fresh tonsil tissue. Tonsil tissue was obtainable on a sporadic basis from the surgical pathology department and large numbers of cells could be obtained from a single piece of tissue. Considerably less virus bound to normal B cells than to Raji cells. However, even though the increase in fluorescence measured with R_{18}-labeled virus bound to normal B cells was smaller than that measured with lymphoblastoid cells, a measurable signal was obtained. The increase in fluorescence expressed as a percentage of the maximum obtainable after addition of Triton was less than that seen in experiments with lymphoblastoid cells (Figure 2-6).

In order to rule out the interference of monocyte engulfment of virus in the determination of maximum relief of fluorescence, experiments were done using cells

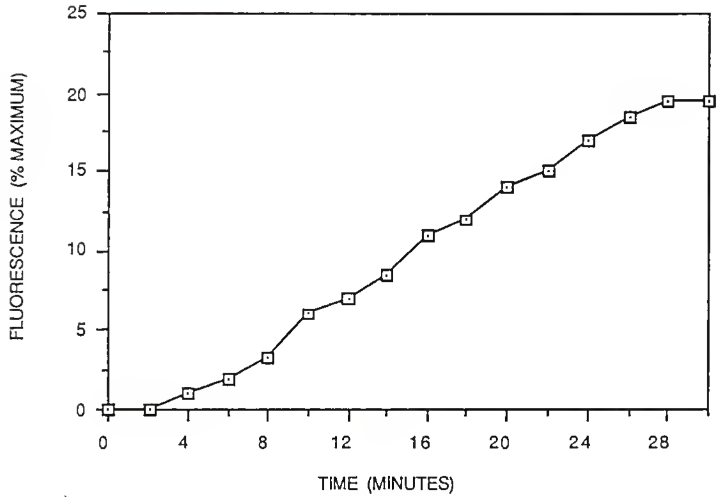


Figure 2-6. Relief of self-quenching of R_{18}-labeled P3HR1-Cl13 virus bound to tonsil derived T-depleted leukocytes expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

that had been depleted of monocytes. Table 2-4 shows the extent of monocyte depletion as determined by cell counts and nonspecific esterase stain pre and post depletion. Figure 2-7 shows the increase in fluorescence of tonsil derived B cells pre and post monocyte depletion by adherence to plastic. The cell preparations treated with iron filings could not be used in the fluorometer due to scatter interference from residual filings in the preparation. The maximum increase in fluorescence achieved with tonsil derived B cells was 20-23% and depletion of monocytes from the cells used did not affect this measurement.

Fusion experiments were also done using T depleted peripheral leukocytes. Human peripheral leukocytes could be obtained with greater regularity than tonsil tissue. Figure 2-8 shows data obtained using T depleted peripheral leukocytes. As seen with the tonsillar B cells, less virus bound peripheral B cells than Raji cells, but the maximum increase in fluorescence was higher than the level obtained with tonsil derived cells. In this experiment the maximum increase was 55%, in other experiments using different batches of labeled virus and different cells, values ranging from 28-56% were achieved.

Changes in Fluorescence of R_{18}-labeled Virus with EBV-Immortalized B Cells

Human B cells were infected with EBV and were immortalized. These cells, designated BAT, have growth characteristics of a continuous cell line, but since they are recently immortalized, they may be biologically more similar to B cells than the lymphoblastoid cell lines, such as Raji, which has been in culture for many years. Raji cells have been reported to have alterations in the cytoskeleton (Bachvaroff et al., 1980). Figure 2-9 demonstrates how these cells function in the fluorescence dequenching assay. Utilizing these cells reduces the need to obtain fresh human

Table 2-4. Monocyte depletion of T-depleted human leukocytes by adherance to plastic.

Cell treatment	cell number	% cells staining esterase positive[1]
none	3.0×10^8	45%
adherance	1.8×10^8	8%

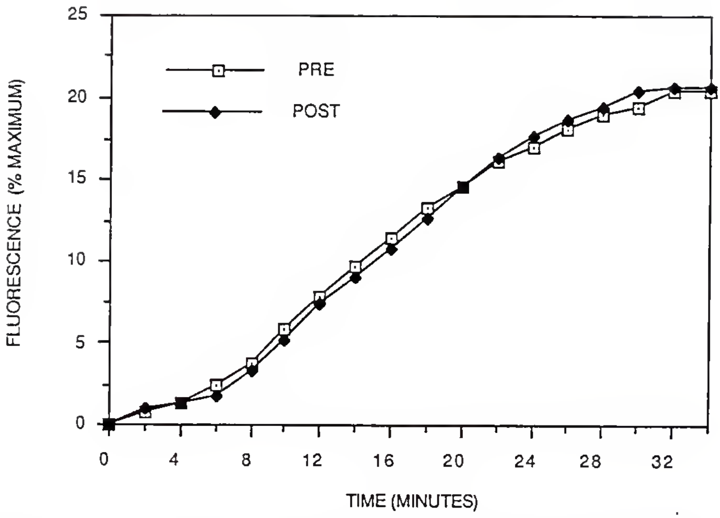


Figure 2-7. Comparison of relief of self-quenching of R_{18}-labeled P3HR1-Cl13 virus bound to tonsil derived B cells pre and post monocyte depletion by adherance to plastic. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

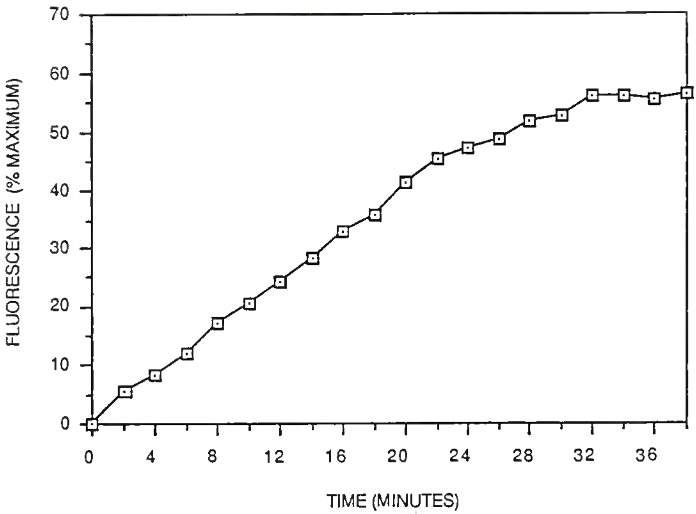


Figure 2–8. Relief of self-quenching of R_{18}-labeled MCUV5 virus bound to fresh T-depleted peripheral leukocytes expressed as a percent of the maximum release obtained after addition of Triton X–100 (infinite dilution).

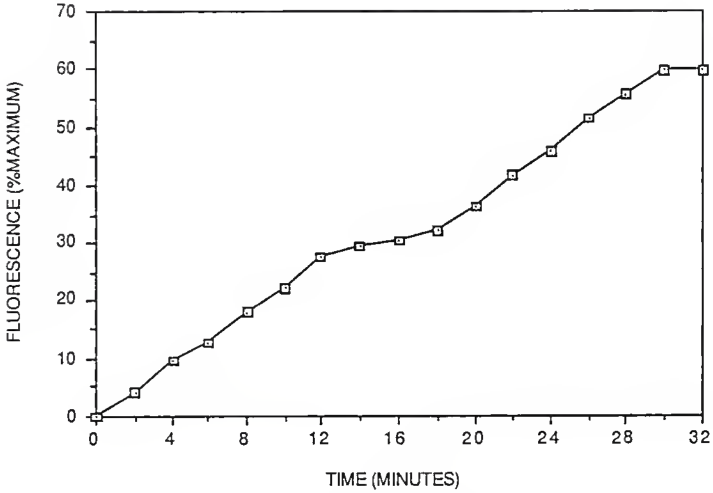


Figure 2-9. Relief of self-quenching of R_{18}-labeled MCUV5 virus bound to BAT cells expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

peripheral blood for each experiment. In future experiments virus entry into BAT cells will be studied in parallel and compared to entry into fresh normal B cells.

Discussion

The fluorescent amphiphile octadecyl rhodamine B chloride (R_{18}) has been used by several groups to study interactions of virus with biological membranes and liposomes (Blumenthal et al., 1987; Gilbert et al., 1990; Hoekstra et al., 1984, 1985; Lapidot et al., 1987; Morris et al., 1989; Sinangil et al., 1988; Stegmann et al., 1986; Wunderli-Allenspach and Ott, 1990). The results from these papers indicate that fluorescence dequenching reflects the occurrence of virus membrane fusion and when discussing the results of the experiments in this dissertation, fluorescence dequenching and membrane fusion will be considered interchangeable terms. The behavior of R_{18}-labeled EBV, as demonstrated by relief of self-quenching of virus bound to Raji cells, and the absence of fluorescence of virus bound to fixed Raji cells or Molt 4 cells, provides strong corroborative support for this conclusion. Fixed cells are resistant to virus membrane fusion (Gilbert et al., 1990; Lapidot et al., 1987) and Molt 4 cells are reported to bind but not internalize virus (Menezes et al., 1977).

The R_{18} labeling procedure did not affect the binding specificity or the amount of EBV that bound to lymphoblastoid cells. This is in agreement with the effect of labeling on attachment of Sendai virus (Hoekstra et al., 1985). Labeling of VSV with R_{18} has been reported to enhance virus binding by twofold, possibly because the probe is positively charged and increases the net charge of the virus (Blumenthal et al., 1987). Labeled virus retained its infectivity as indicated by its ability to induce immunoglobulin synthesis by cultured T-depleted peripheral leukocytes.

The early events in infection of normal B cells and lymphoblastoid cells have been examined previously by electron microscopy (Nemerow and Cooper, 1984a; Seigneurin et al., 1977). These studies reported that EBV enters lymphoblastoid cells by direct fusion with the outer cell membrane and that virus is endocytosed into thin-walled non-clathrin coated vesicles in the normal B cell before it fuses with the cell membrane. Both pathways were reported to initiate within two to five minutes at 37°C. The kinetics of fusion with Raji cells, normal lymphocytes, and recently immortalized BAT cells were very similar, all exhibiting a measurable change within two minutes of warming in the cuvette of the spectrofluorometer. A one to two minute lag time, corresponding to the time required for initial entry of ligands, toxins, and virions into an acidic compartment after receptor mediated endocytosis (Bridges et al., 1982) has been reported for relief of self-quenching of R_{18}-labeled vesicular stomatitis virus bound to Vero cells (Blumenthal et al., 1987).

Since EBV appears capable of fusing with the plasma membrane at the cell surface, or after endocytosis, this may mean that either virus can enter normal B cells by both routes, or that fusion with an endocytic vesicle wall occurs rapidly after uptake, perhaps even before virus is exposed to low pH. It has been shown that rotavirus enters cells by direct cell membrane penetration (Kaljot et al., 1988) even though earlier electron microscopy studies had revealed presence of rotavirus particles in coated pits and a variety of vesicles, signifying entry by endocytosis (Petrie et al., 1981; Quann and Doane, 1983).

Experiments using lysosomotropic agents, inhibitors of endocytosis, and pH sensitive fluorescent probes may help answer the question of whether EBV is capable of fusing at both the plasma membrane and the endocytic vesicle of lymphocytes. In

1984, Nemerow and Cooper demonstrated a 96% reduction in infectivity by EBV of B cells treated with 1mM chloroquine and a 20% reduction in infectivity of cells treated with 10mM NH_4Cl. Infectivity was assessed by stimulation of host cell DNA synthesis as measured by incorporation of $[^3H]$ thymidine after 4 to 6 days in culture. From their studies they concluded that a reduction in pH was necessary for virus entry because of inhibition by these agents. The fluorescence dequenching assay allowed for analysis of the effects of these reagents on EBV fusion and the results are presented and discussed in the following chapter.

CHAPTER 3
EFFECTS OF LYSOSOMOTROPIC AGENTS AND pH ON FUSION OF EPSTEIN-BARR VIRUS WITH LYMPHOCYTES

Introduction

To initiate an infection, all enveloped animal viruses must fuse with a cellular membrane and this fusion can be divided into two general classes, low pH dependent and pH independent. It is generally considered that viruses that are low pH dependent fuse from within acidic vesicles whereas viruses that are low pH independent can fuse directly with the plasma membrane, but may fuse from endosomes as well. Although fusion of EBV with lymphoblastoid cell lines occurs at the plasma membrane and therefore presumably does not require exposure to low pH, virus has been reported to fuse with normal B cells after endocytosis and certain lysosomotropic agents have been shown to inhibit virus infectivity (Nemerow and Cooper, 1984a). The possibility that penetration of Epstein-Barr virus nucleocapsids into the cytosol might involve an acid-catalyzed fusion reaction in the endosomal compartment was further investigated in this work since our assay measures membrane fusion in isolation of other events in the virus life cycle that might be affected by drugs. Exposure to pH values between 5.0 and 7.0 has dramatic effects on many of the molecules brought into the cell by endocytosis. Many ligands dissociate from their receptors at pH values below 7.

Some viruses undergo significant changes in conformation when exposed to acidic pH (White, 1990). The fusion glycoprotein of influenza virus, the hemagglutinin

(HA) undergoes an irreversible conformational change upon exposure to mildly acidic pH within acidic organelles after endocytosis. If virus is bound to the cell surface and the extracellular pH is briefly lowered to pH 5.0, fusion of the virus can occur at the plasma membrane. If the virus alone is exposed to acidic pH the conformation occurs prematurely and the virus is unable to fuse. Treatment of cells with lysosomotropic agents inhibited influenza infectivity. The unprotonated form of these lipophilic amines crosses cell membranes but the protonated form does this far less efficiently. When the uncharged form enters acidic compartments it becomes protonated, thereby raising the pH and inhibiting its own escape across the membranes of the vacuoles.

Vesicular stomatitis virus (VSV) is another example of a virus that fuses from within an acidic compartment after endocytosis (Dahlberg, 1974; Dales, 1973; Dickson et al., 1982; Matlin et al., 1982). The fusion activity can be shown to take place on the plasma membrane if cells with VSV attached to their surfaces are placed in pH 5.9 medium (Matlin et al., 1982; Blumenthal et al., 1987). Lysosomotropic agents were also shown to inhibit fusion from with an endocytic vesicle, but had no effect on fusion at the plasma membrane at pH 5.9 (Blumenthal et al., 1987).

The work described here sought to determine whether EBV fusion is a truly pH dependent event and where fusion takes place in lymphoblastoid cell lines, freshly isolated human B cells and recently transformed human B cells.

Materials and Methods

Membrane Fusion Assay

Virus that has been labeled with R_{18} at self-quenching concentration was added to 2 X 10^6 cells and incubated for 1 hour on ice in the dark. Cells were washed four

times with ice-cold Dulbecco's saline at pH 7.4 and suspended in 400ul of Dulbecco's at pH 7.4 (unless otherwise indicated) when transferred to the microcuvette of a spectrofluorometer (SLM SPF 500C, SLM Instruments Co., Urbana, Illinois) equipped with a magnetic stirrer and circulating water bath set at 37°C. Fluorescence dequenching was monitored continuously at an excitation wavelength of 560nm and an emission wavelength of 585nm. At the end of the assay, Triton X-100 (1% v/v, final concentration) was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore.

Cells

The lymphoblastoid cell lines Raji (Pulvertaft, R.J., 1964) and BAT, which are both EBV genome-positive human B-cell lines expressing the virus receptor CR2 (CD21); Molt 4 (Minowda et al., 1972), an EBV genome negative human T cell line that expresses CR2, but cannot internalize virus (Menezes et al., 1977) and P3HR1-Cl5 (Heston et al., 1982), an EBV genome-positive human B-cell line which does not express CR2 were grown at 37°C and diluted at least biweekly in RPMI 1640 supplemented with heat-inactivated fetal calf serum, 100IU of penicillin and 100ug of streptomycin per ml. Fresh human T cell-depleted leukocytes were isolated as described previously from peripheral blood and used directly in assays.

Treatment of Cells with Lysosomotropic Agents

Ammonium chloride (NH_4Cl), chloroquine, and methylamine were purchased from Sigma and stock solutions were made in phosphate buffered saline of 100mM, 100mM, and 50mM respectively, from which dilutions were made in RPMI 1640 for incubation with cells. Cells were incubated in one milliliter of media containing the lysosomotropic agent for 35 minutes at 37°C to neutralize acidic intracellular

compartments and control cells were incubated in medium only. At the end of the incubation the cells were pelleted by centrifugation and the supernatant was removed. Cells were resuspended in 100ul of media and incubated with virus for 1 hour on ice.

Determination of Intracellular pH

To determine intracellular pH, cells were incubated with a mixture of fluorescein isothiocyanate (FITC) and tetramethylrhodamine (TRITC)-labeled dextrans (70,000 mw) (Molecular Probes Inc., Junction City, Oregon) for 35 minutes at 37°C to allow uptake of the labeled dextrans into the cells. Cells were washed free of unassociated dextran and analyzed in the spectrofluorometer by measuring the TRITC fluorescence at an excitation wavelength of 560nm and an emission wavelength of 580nm followed by measuring FITC emission at 522nm at excitation wavelengths from 450nm to 518nm. Monensin (1ug/ml) was then added to equilibrate extracellular (test pH) and endosomal pH and another fluorescence measurement of the FITC was taken from 450nm to 518nm. If the intensity of the fluorescence rises at this step, the average pH of the intracellular compartments is below the test pH. If the intensity falls, the average pH was above the test pH.

Incorporation of 5-(N-octadecanoyl)aminofluorescein into Virus Membranes

The membrane probe 5-(N-octadecanoyl)aminofluorescein (AF) is a fluorescent amphiphile containing a long hydrocarbon chain which allow it to insert readily into biological membranes (Figure 3-1). AF manifests the same property of concentration-dependent quenching of fluorescence as R_{18}, in addition to sensitivity to changes in pH similar to the FITC-dextran used for determination of intracellular pH. A stock solution of 50mg/ml of AF (Molecular Probes, Inc., Junction City, Oregon) was prepared in dimethylformamide and stored at -20°C. The probe was incorporated into

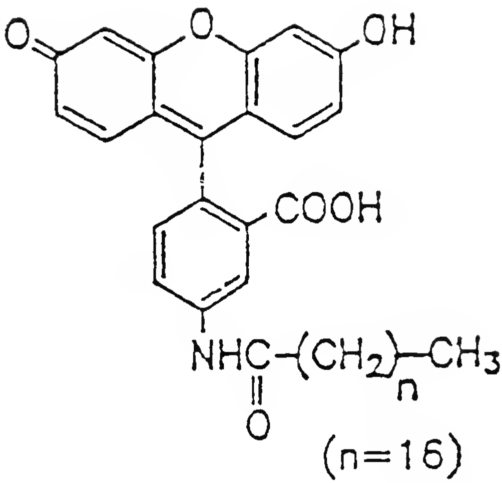


Figure 3-1. Structural formula of 5-(N-octadecanoyl)aminofluorescein (AF).

virus membranes by modification of the method used to incorporate R_{18} into virus membranes. Briefly, 2ul of the stock AF was added to 250ul of the MCUV5 strain of EBV that had been collected from culture supernatant and concentrated 250-fold. Virus and AF were vortexed immediately after addition of the fluorescent probe and incubated at room temperature in the dark for 1 hour. Virus and non-incorporated AF were separated by chromatography on Sephadex G-75 (Sigma Chemical Co., St. Louis, Missouri) recovering the AF-labeled virus in the void volume. Labeled virus was aliquoted and stored at -70°C.

Fluorescence measurements were made at an excitation wavelength of 496nm and an emission wavelength of 522nm using a SLM SPF500c spectrofluorometer equipped with a thermostatically-controlled cuvette chamber and magnetic stirrer (SLM Aminco, Urbana, Illinois).

Results

Effect of Lowering Extracellular pH on Fusion

Previous studies with viruses that are known to be dependent on the low pH of the endosome in order to fuse have shown that they are also able to fuse at the plasma membrane if the pH of the extracellular medium if briefly lowered (Blumenthal et al., 1987; Marsh et al., 1983a; White et al., 1980). Experiments were therefore done to see if the rate or extent of fusion of EBV would be affected if the pH of the extracellular media was decreased in order to drive low pH-dependent fusion to occur at the plasma membrane. The results in Figures 3-2, 3-3, and 3-4 indicate that altering the extracellular pH from 7.4 to 5.5 did not effect virus fusion with Raji, BAT, or fresh T-depleted leukocytes.

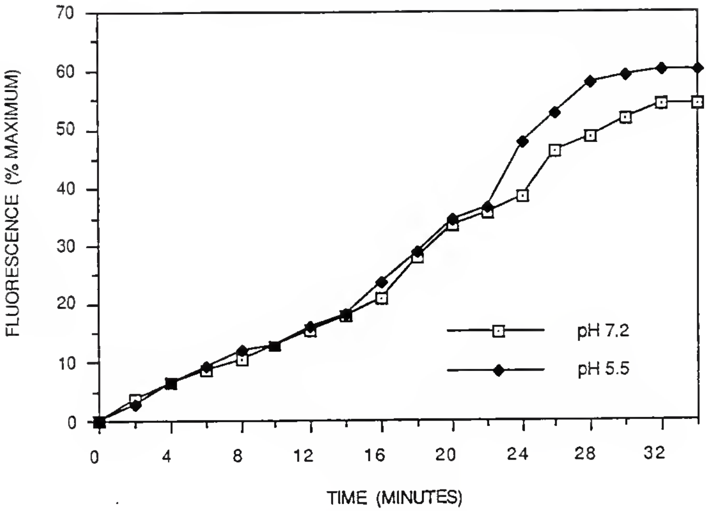


Figure 3-2. Relief of self-quenching of R_{18}-labeled MCUV5 virus bound to Raji cells at pH 7.2 or pH 5.5. Virus was bound to cells at pH 7.2, cells were washed to remove unbound virus and cells were resuspended in pH 7.2 or pH 5.5 Dulbecco's saline. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

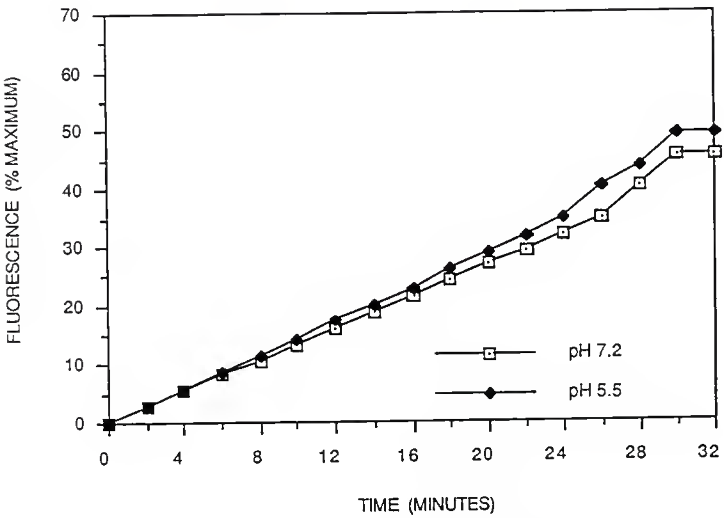


Figure 3-3. Relief of self-quenching of R_{18}-labeled MCUV5 virus bound to BAT cells at pH 7.2 or pH 5.5. Virus was bound to cells at pH 7.2, cells were washed to remove unbound virus and cells were resuspended in pH 7.2 or pH 5.5 Dulbecco's saline. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

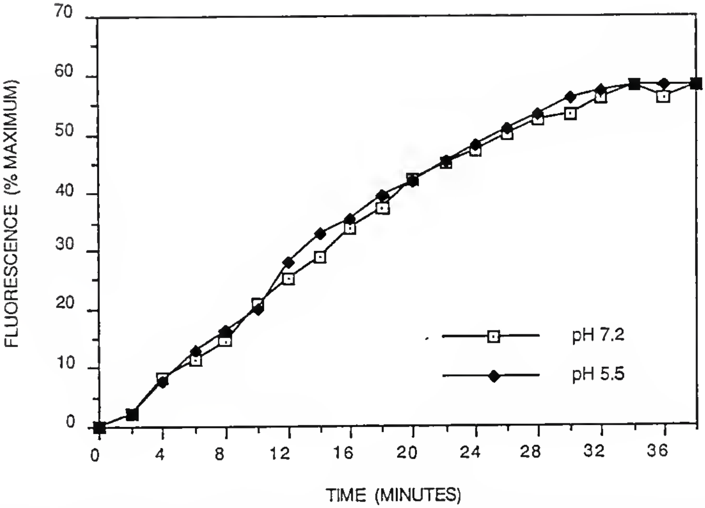
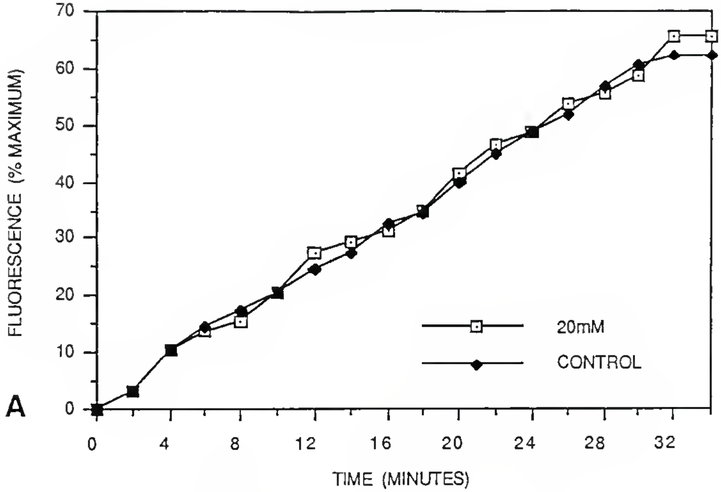


Figure 3-4. Relief of self-quenching of R_{18}-labeled MCUV5 virus bound to T-depleted leukocytes at pH 7.2 or pH 5.5. Virus was bound to cells at pH 7.2, cells were washed to remove unbound virus and cells were resuspended in pH 7.2 or pH 5.5 Dulbecco's saline. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

<u>Effect of Lysosomotropic Agents on Virus Fusion</u>

Although fusion of EBV with lymphoblastoid cell lines occurs at the plasma membrane and therefore presumably does not require exposure to low pH, virus has been reported to fuse with normal B cells after endocytosis and certain lysosomotropic agents capable of altering the pH of intracellular compartments have been shown to inhibit virus infectivity (Nemerow and Cooper, 1984a). These agents have been used in many virus systems to determine the mechanism by which virus enters cells (Andersen and Nexo, 1983; Blumenthal et al., 1987; Cassel et al., 1984; Gilbert et al., 1990; Gollins and Porterfield, 1986; Stein et al., 1987).

The effects of ammonium chloride (NH_4Cl), methylamine and chloroquine on three cell types, Raji, BAT, and fresh T-depleted human leukocytes were studied in the fluorescence dequenching assay. Figures 3-5, 3-6 and 3-7 demonstrate that 20mM NH_4Cl did not have any effect on fusion of virus with any of the three cell types. Three concentrations of chloroquine were tested with Raji cells and did not effect fusion (Figure 3-8). In contrast, chloroquine BAT cells and fresh T-depleted leukocytes exhibited dose-dependent inhibition of fluorescence dequenching shown in Figures 3-9 and 3-10. Chloroquine inhibited fusion of virus with BAT cells by 34% at 1mM and by 30% at 0.5mM. For peripheral B cells, the inhibition was 60% at 1mM, 50% at 0.5mM, and 24% at 0.2mM. The third agent used, methylamine, which in addition to elevating the endosomal pH also is an inhibitor of transglutaminase which has been suggested to be involved in receptor-mediated endocytosis (Davies et al, 1980), did not inhibit relief of self-quenching with any of the three cell types (Figure 3-11), thus paralleling the data for the NH_4Cl-treated cells. In confirmation that these



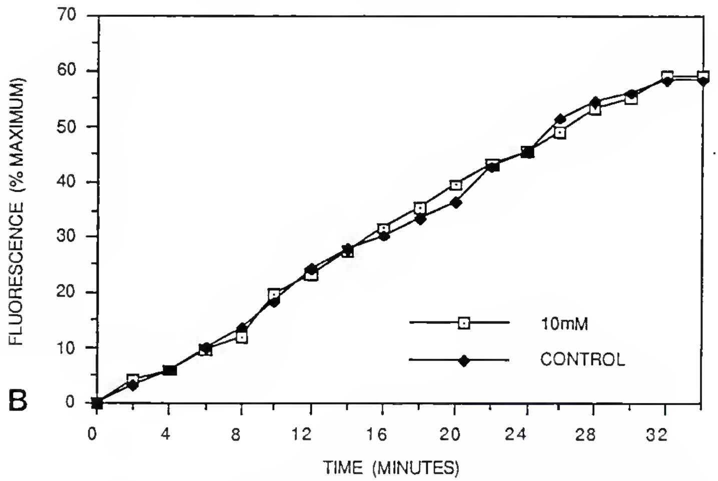


Figure 3-5. Effect of preincubation of Raji cells with ammonium chloride (NH_4Cl) or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Panel A, 20mM NH_4Cl; panel B, 10mM NH_4Cl. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

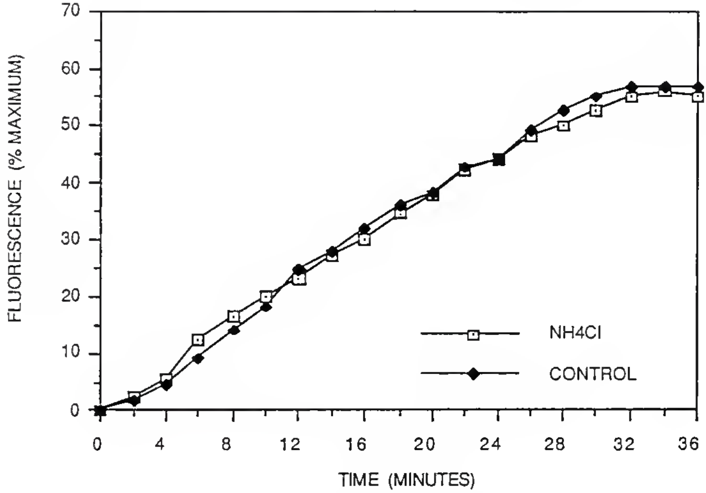


Figure 3-6. Effect of preincubation of BAT cells with 20mM ammonium chloride (NH_4Cl) or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

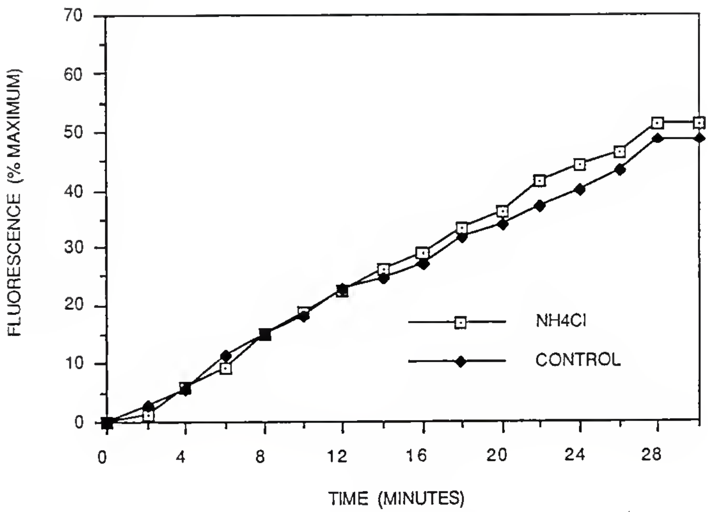


Figure 3-7. Effect of preincubation of T-depleted leukocytes with 20mM ammonium chloride (NH_4Cl) or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

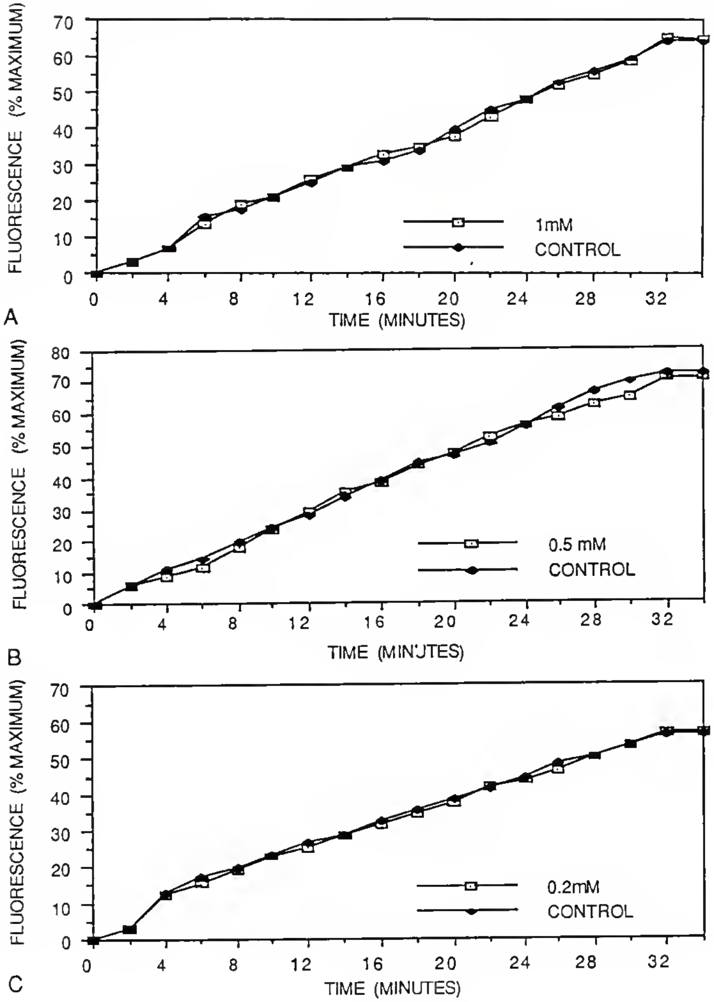


Figure 3-8. Effect of preincubation of Raji cells with chloroquine or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Panel A, 1mM; panel B, 0.5mM; panel C, 0.2mM. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

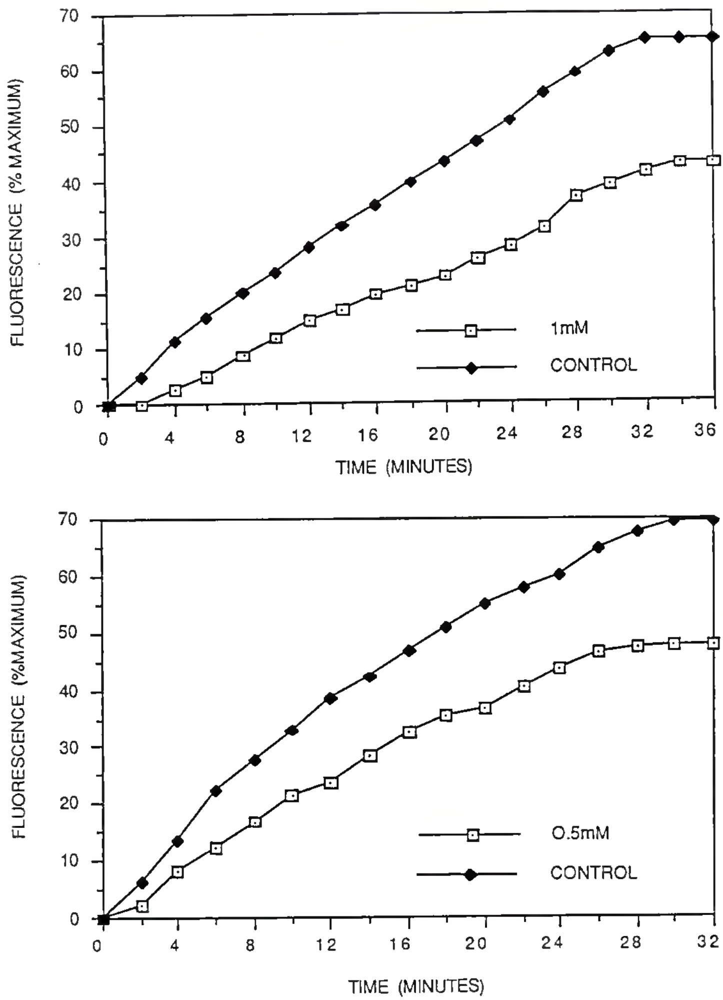


Figure 3-9. Effect of preincubation of BAT cells with chloroquine or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Panel A, 1mM; panel B, 0.5mM. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

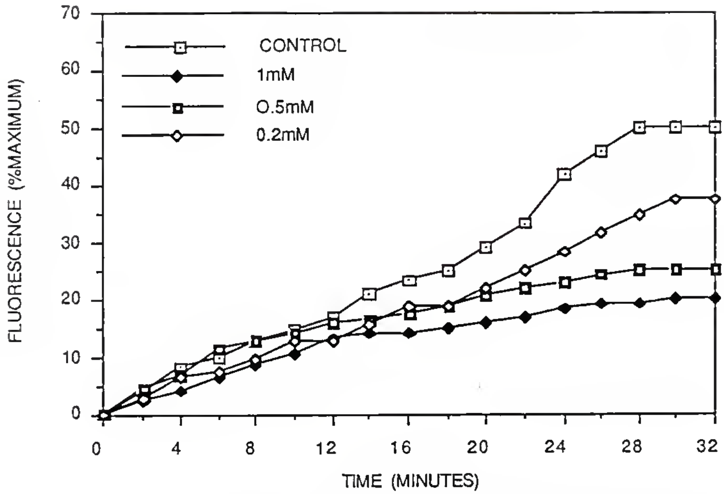


Figure 3-10. Effect of preincubation of T-depleted leukocytes with chloroquine or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

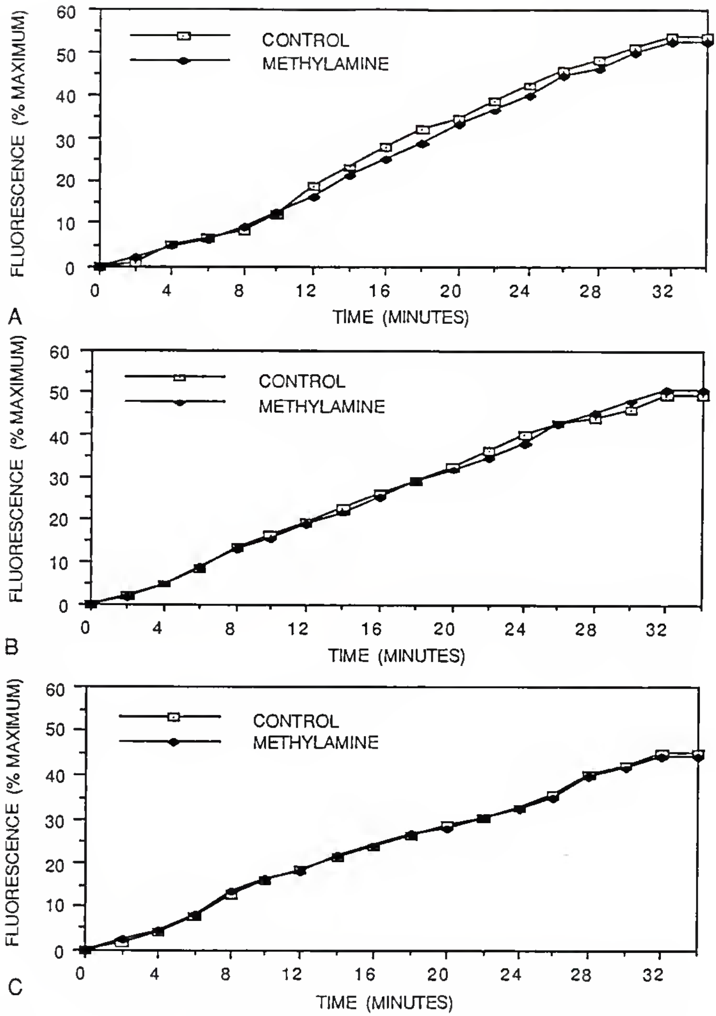


Figure 3-11. Effect of preincubation of Raji cells (panel A), BAT cells (panel B), and T-depleted leukocytes (panel C) with 5mM methylamine or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton-X-100 (infinite dilution).

agents were indeed increasing the intracellular pH, the resulting pH after treatment of the cells was determined.

Determination of pH of Intracellular Compartments After Treatment with Lysosomotropic Agents

The pH in endocytic compartments can be measured using fluorescein-labeled ligands such as dextran (Ohkuma and Poole, 1978; Tyoko and Maxfield, 1982; Yoshimura and Ohnishi, 1984.). The fluorescence intensity of fluorescein decreases dramatically between pH 7.0 and pH 5.0. Thus, changes in fluorescence intensity can be used as an assay for changes in pH. The ratio of fluorescence intensities at the wavelengths of 450nm and 496nm can be used to determine a standard curve from which actual pH values can be extrapolated (Geisow, M.J., 1984). In various cell types, lysosomes have pH values between 4.6 and 5.2, and endocytic vesicles have pH values between 5.0 and 5.5 (Maxfield and Yamashiro, 1987; Ohkuma and Poole, 1978; Tyoko and Maxfield, 1982; Tyoko et al., 1983; Yamashiro and Maxfield, 1984).

For accurate pH determinations using the fluorescent conjugates it was necessary either to ensure sufficient pinocytosis to produce a reliable signal at 450nm or to collapse the intracellular pH gradients using monensin. Monensin is a carboxylic ionophore which is able to promote exchange of protons for univalent cations and thereby abolishes transmembrane proton gradients (Pressman, 1976; Tartakoff, 1977). After addition of monensin to cells, the fluorescein emission will resemble that expected at the external pH. By altering the external pH, a calibration curve can be obtained of intracellular fluorescein isothiocyanate (FITC)-dextran. In addition to the fluorescein conjugate, a rhodamine conjugate is also included to ensure sufficient uptake of the ligands into the cells. The fluorescence of the rhodamine is insensitive to changes in pH and was used as an internal reference for the amount of conjugate

uptake. Figure 3-12 demonstrates the change in the fluorescence intensity of FITC-dextran in buffer at various pH values. This standard curve can be used in conjunction with the differences in fluorescence seen upon addition of monensin to cells to determination the intracellular pH. After addition of monensin to cells, the fluorescein emission resembles that expected at the external pH. By altering the external pH, the pH of the intracellular FITC-dextran was obtained.

Figure 3-13 shows the fluorescence emission at 522nm of BAT cells that have endocytosed FITC and TRITC-labeled dextran. The value of the fluorescence of the TRITC was recorded as a control value for dextran uptake and compared between samples. Fluorescence measurements were made in pH 7.4 medium before and after addition of monensin. In the initial scan, the FITC emission was very low, indicating that the fluorescence was quenched due to acidic pH, after addition of monensin there was a great change in the fluorescence with a peak at 496nm. The TRITC fluorescence value was 1370 arbitrary units (a.u.). Cells treated with 20mM NH_4Cl were incubated with the labeled dextrans and analyzed for flüorescence before and after addition of monensin. Figure 3-14 (panel A) shows the results when the extracellular medium was pH 7.4. The fluorescence before addition of monensin was much higher than the fluorescence of the untreated cells, indicating that the pH had been elevated. Addition of monensin produced a slightly higher emission pattern, indicating that the internal pH was not at pH 7.4, but that it was higher than the control cells. The TRITC fluorescence was 1405 a.u.. The same assay was performed except with an extracellular medium of pH 7.0 (Figure 3-14, panel B). In this case the fluorescence measurements were essentially equal before and after addition of

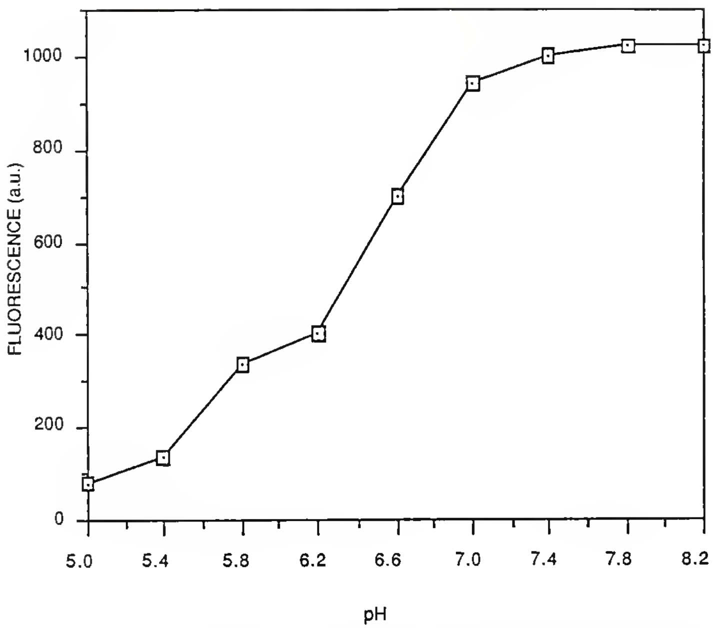


Figure 3-12. Relative intensity of fluorescein isothiocyanate-dextran (FITC-dextran) fluorescence as a function of changes in pH. FITC-dextran was excitated at a wavelength of 496nm and emission was measured at 522nm.

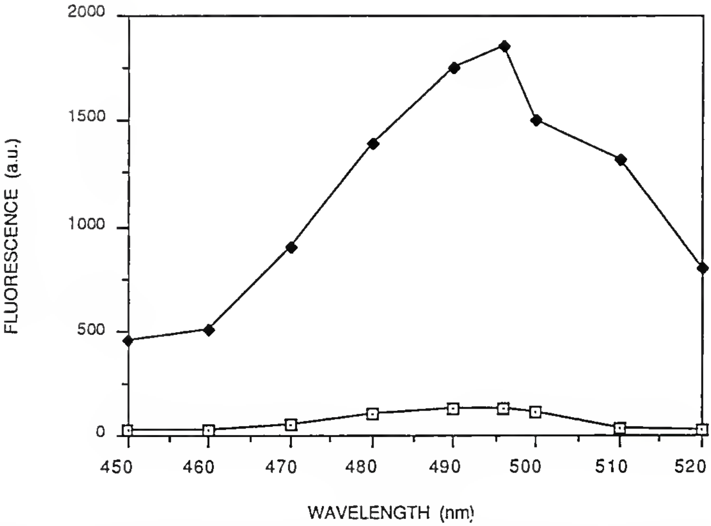


Figure 3-13. Excitation spectra at pH 7.4 of BAT cells containing FITC-dextran before (-□-) and after (-◆-) addition of monensin. Measurements were taken at a fixed emission wavelength of 522nm and fluorescence is expressed in arbitrary units (a.u.).

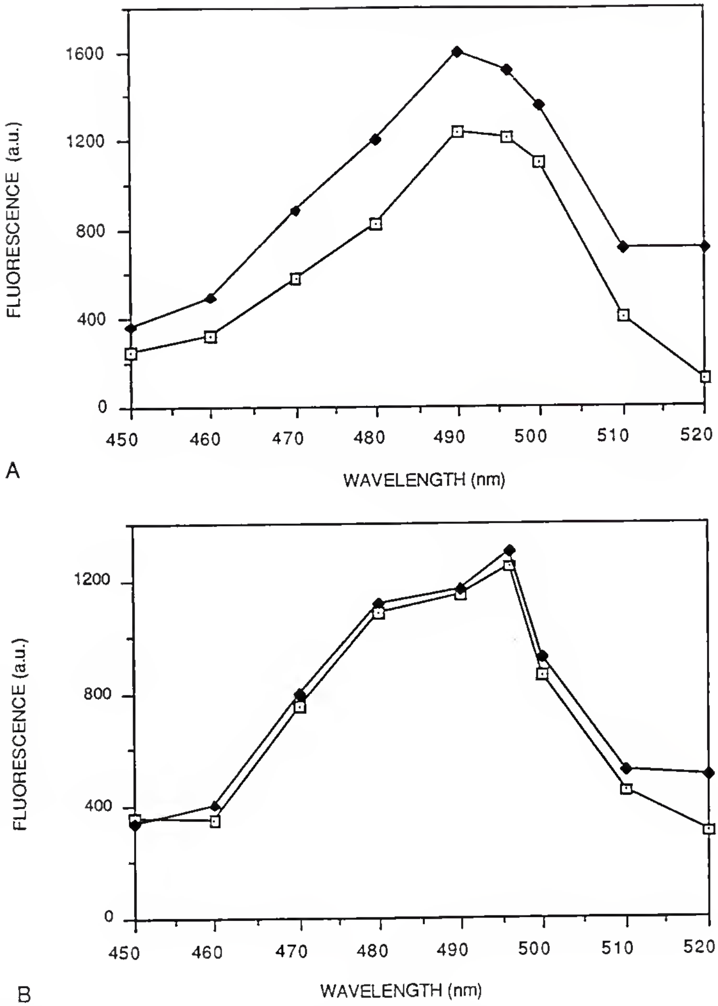


Figure 3-14. Excitation spectra at pH 7.4 (panel A) and pH 7.0 (panel B) of NH_4Cl treated BAT cells containing FITC-dextran before (—□—) and after (—◆—) addition of monensin. Measurements were taken at a fixed emission wavelength of 522nm and fluorescence is expressed in arbitrary units (a.u.).

monensin indicating that the intracellular pH was neutralized to pH 7.0 by the NH_4Cl. The TRITC fluorescence was 1390 a.u..

Cells that were treated with 1mM chloroquine were tested in the same manner as the NH_4Cl treated cells. Figure 3-15 illustrates the results of chloroquine treated cells at external test pH values of pH 7.4. The chloroquine appeared to have elevated the intracellular pH to pH 7.4. since the fluorescence before and after monensin were superimposable. The TRITC fluorescence value was 1540 a.u. for the control cells and 890 a.u. for the chloroquine treated cells. The amount of TRITC fluorescence and FITC fluorescence after addition of monensin were lower for the chloroquine treated cells than the control cells reflecting that there was less uptake of the dextran into the chloroquine treated cells than the control cells. There are reports in the literature of many effects of chloroquine on cells besides elevating the endosomal pH, such as rendering membranes very resistant to mechanical stress; also that chloroquine reduces uptake of some ligands into cells (Matsuzawa and Hostetler, 1980; Wibo and Poole, 1974).

Cells treated with 5mM methylamine were also tested for the ability of this agent to neutralize intracellular compartments. The results with this agent paralleled the results with NH_4Cl treated cells, the methylamine elevates the intracellular pH to 7.0 (Figure 3-16). The values for the TRITC fluorescence were 1480 a.u. for the control and 1465 a.u. for the methylamine treated cells.

Fluorescence Dequenching of AF-labeled EBV

Virus that was labeled with AF was shown to be infectious by the same methods used for R_{18}-labeled EBV (Table 3-1). Figure 3-17 demonstrates the fluorescence properties of virus labeled with AF as a function of changes in pH. The

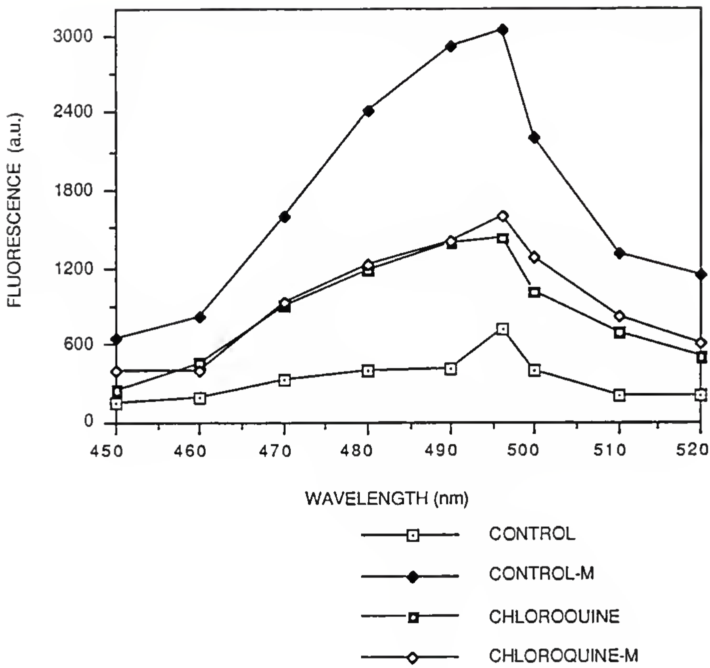


Figure 3-15. Excitation spectra at pH 7.4 of chloroquine treated and untreated (control) BAT cells containing FITC-dextran before and after addition of monensin (M). Measurements were taken at a fixed emission wavelength of 522nm and fluorescence is expressed in arbitrary units (a.u.).

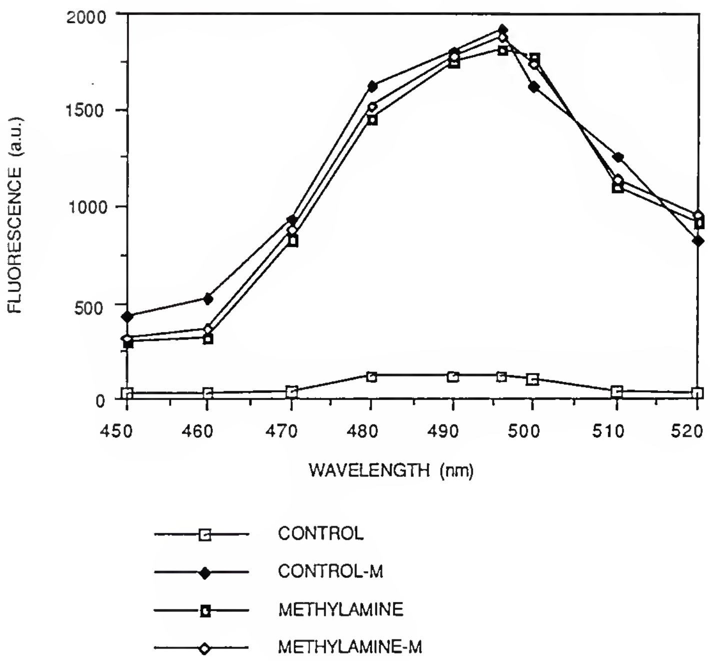


Figure 3-16. Excitation spectra at pH 7.0 of methylamine treated and untreated (control) BAT cells containing FITC-dextran before and after addition of monensin (M). Measurements were taken at a fixed emission wavelength of 522nm and fluorescence is expressed in arbitrary units (a.u.).

Table 3-1. Effect of labeling with AF on the ability of MCUV5 virus to induce immunoglobulin synthesis by fresh T-depleted human leukocytes.

Virus dilution	Immunoglobulin conc. ng/ml with:	
	AF-labeled virus	Mock-labeled virus
1/20	14,259	18,265
1/40	28,425	24,579
1/80	32,675	31,469
1/160	34,996	37,904
1/320	28,490	30,246
1/640	21,245	24,170
1/1280	12,248	10,960
none	1,438	

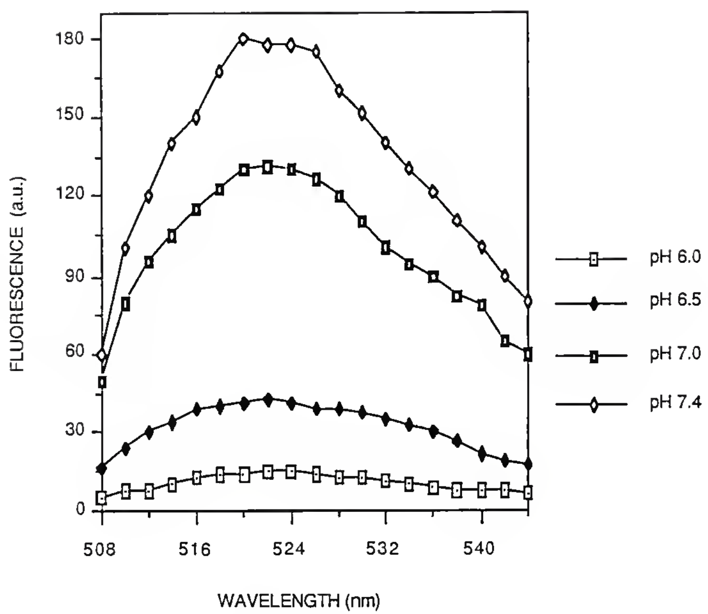


Figure 3-17. Fluorescence properties of virus labeled with AF at pH 6.0 to pH 7.4. Measurements were taken at an excitation wavelength of 496nm. Fluorescence intensity is expressed in arbitrary units (a.u.).

fluorescence is very sensitive to changes below pH 7.0 and is essentially undetectable below pH 6.0.

The first cell types to be tested with AF-labeled EBV were Raji and Molt-4 cells. Virus fuses with the Raji cell at the plasma membrane, therefore the fluorescence should not be subject to a low pH environment. The Molt-4 cells are able to bind virus but the virus does not penetrate the cell membrane. The data in Figure 3-18 show fluorescence dequenching up to 45% of the total bound to Raji cells but only 5.2% relief of quenching of virus bound to Molt-4 cells. Raji cells were also tested using media with a pH of 5.5. The fluorescence remained quenched over a time course of 32 minutes and the fluorescence when Triton was added the fluorescence was also quenched (Figure 3-19). The fluorescence could be unquenched by addition of 1.0M sodium phosphate to the cuvette, thus allowing determination of the amount of fluorescence that bound to the cells.

The fluorescence dequenching of virus bound to BAT cells was very different from that seen with the Raji cells. Figure 3-20 shows a plot of the amount of dequenching of virus bound to cells expressed as a percentage of the value upon addition of Triton. Also shown on the same graph is the percent dequenching of R_{18}-labeled EBV bound to the same population of cells. This difference reflects the inability to measure the fluorescence of the AF-labeled virus that was in an acidic environment.

In order to confirm this hypothesis, BAT cells were treated with 20mM NH_4Cl as done in previous experiments in order to neutralize the acidic vesicles and then these cells were used in fusion assays with AF-labeled EBV. Figure 3-21 illustrates the results of this experiment. Virus fusion could be measured in the NH_4Cl treated cells

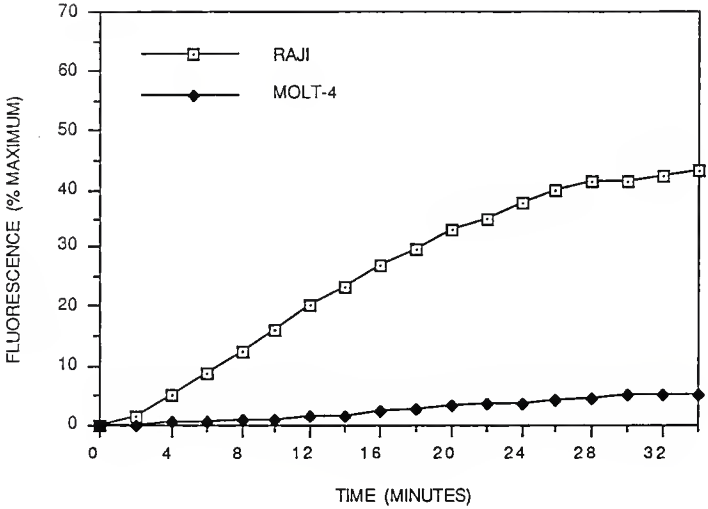


Figure 3-18. Relief of self-quenching of AF-labeled MCUV5 virus bound to Raji and Molt 4 cells. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

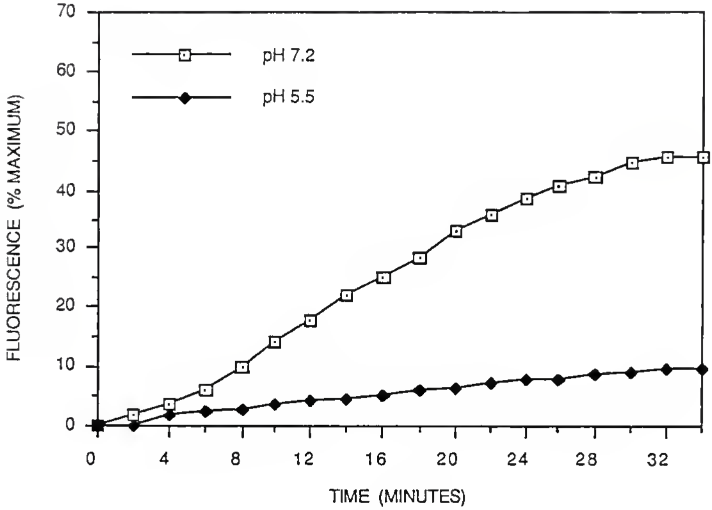


Figure 3-19. Relief of self-quenching of AF-labeled MCUV5 virus bound to Raji cells at pH 7.2 or pH 5.5. Virus was bound to cells at pH 7.2, cells were washed to remove unbound virus and cells were resuspended in pH 7.2 or pH 5.5 Dulbecco's saline. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

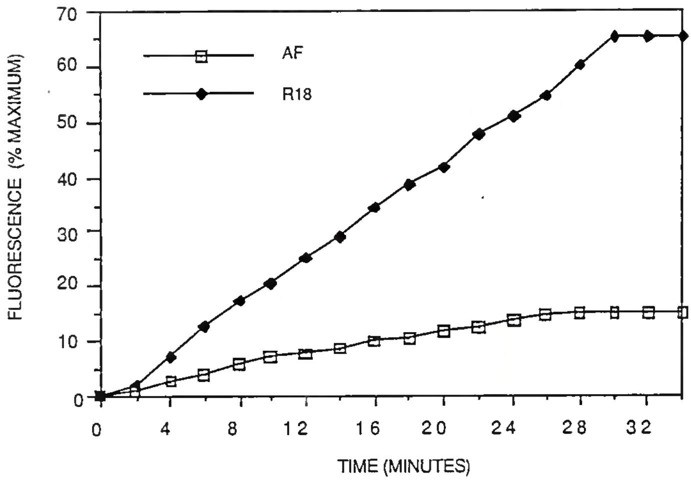


Figure 3-20. Relief of self-quenching of AF-labeled or R_{18}-labeled MCUV5 virus bound to BAT cells at pH 7.2. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

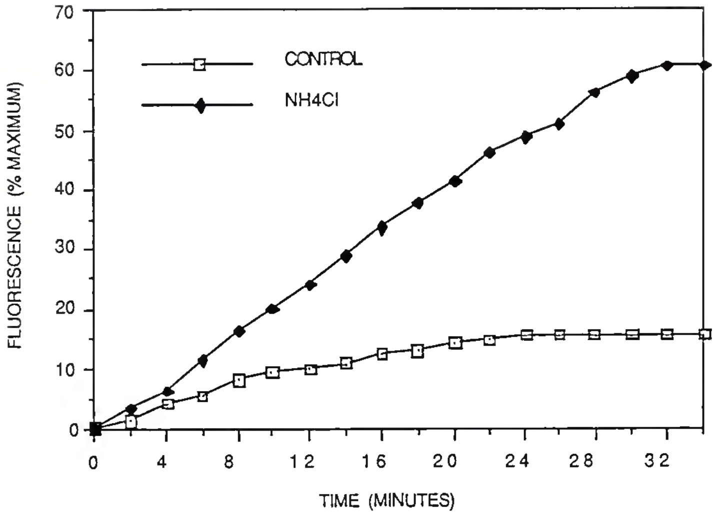


Figure 3-21. Effect of preincubation of BAT cells with ammonium chloride (NH_4Cl) or RPMI on relief of self-quenching of AF-labeled MCUV5 virus bound to cells at pH 7.2. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

to an extent comparable to that found with R_{18}-labeled virus, whereas in the untreated cells the virus fusion was marginally detectable due to the pH-dependent quenching of the fluorophore. These data not only show that most of the virus fused from within an acidic compartment, but that virus was not dependent upon acidic pH in order to fuse and thus enter the cell to continue the infectious cycle.

Discussion

It is well established that enveloped viruses enter their host cells by membrane fusion, either at the plasma membrane or from within an endocytic vesicle. In many instances, virus entry from within an endocytic vesicle is catalyzed by the acidic environment of the endosome (Blumenthal et al., 1987; White, 1990) and this acidic environment is a requirement for successful virus entry into the cytoplasm of the cell. In order to assess which conditions are necessary for entry of Epstein-Barr virus into lymphocytes, the effects of lysosomotropic agents and low pH treatment were examined on fusion between virus and cellular membranes.

Altering the extracellular medium to pH 5.5 did not result in any increase in the rate or extent of fusion of virus with the lymphoblastoid cells line Raji, the recently EBV-transformed B cell line BAT, or with fresh peripheral T-depleted leukocytes. Viruses that are catalyzed by acidification fuse rapidly and efficiently once in the endosome and this environment can be imitated at the cell surface by lowering the pH of the extracellular medium. If viruses in this category, such as influenza, Semliki Forest, and West Nile virus are acidified before binding to their target membranes, their fusion activity is irreversibly inactivated, presumably due to premature triggering of the acid-activated conformational change in the viral fusion protein necessary for fusion to occur.

Lysosomotropic agents raise the pH of endosomes and they have been shown to inhibit the infectivity of all enveloped viruses tested that display low pH-dependent fusion (Marsh and Helenius, 1989). The three agents tested in this work, chloroquine, NH_4Cl and methylamine failed to inhibit fusion of virus with lymphoblastoid cells. For the lymphoblastoid cells tested, Raji, virus has been reported to gain entry via fusion at the plasma membrane (Nemerow and Cooper, 1984a), so it is not surprising that these agents had no effect on virus fusion. Methylamine and NH_4Cl did not inhibit virus fusion with BAT cells or fresh peripheral T-depleted leukocytes.

In contrast, chloroquine inhibited fusion of virus with BAT cells by 34% at 1mM and by 30% at 0.5mM. For peripheral B cells, the inhibition was 60% at 1mM, 50% at 0.5mM, and 24% at 0.2mM. These results raise the questions of whether chloroquine is effecting other biological processes besides raising the endosomal pH and also whether methylamine and NH_4Cl are effectively neutralizing the acidity of the endocytic compartments. The intracellular pH of BAT cells was determined to be raised to pH 7.0 by treatment with 20mM NH_4Cl or 5mM methylamine and to pH 7.4 by treatment with chloroquine. These data rule out the possibility that these agents were not effectively raising the intracellular pH. Electron microscopy studies of internalization of EBV into chloroquine-treated B lymphocytes showed that formation of endocytic vacuoles proceeded normally in comparison to untreated cells but that in chloroquine-treated B cells, intact virions remained in the vacuoles and very few nucleocapsids were observed in the cytoplasm (Nemerow and Cooper, 1984a). Virus entry into methylamine or NH_4Cl treated cells was not evaluated by electron microscopy by these investigators, presumably because these agents had minimal effect on virus infectivity as determined by stimulation of DNA synthesis, whereas chloroquine

reduced infectivity by 96%. Treatment of cells with methylamine or NH_4Cl is considered an acceptable way of establishing whether a virus is dependent on low pH for fusion. The results with chloroquine treated cells indicate that further studies are necessary to determine the mechanism by which this agent inhibits virus entry into B lymphocytes. Modifications of the endocytic pathway will be investigated in addition to investigating other effects of the drug besides elevating endosomal pH.

Further confirmation that fusion of EBV with B lymphocytes is a pH-independent event was obtained from fusion assays of virus labeled with the pH-sensitive probe 5-(N-octadecanoyl)aminofluorescein (AF). Fusion could be measured in equal amounts with AF or R_{18}-labeled EBV and Raji cells because the virus fused at the plasma membrane with this cell type. Molt cells, which bound virus but did not internalize virus, were negative for fluorescence dequenching of the bound AF-labeled virus indicating that the probe did not exchange between membranes in a non-specific manner. Fusion of AF-labeled virus could not be measured with BAT cells unless the cells were treated with NH_4Cl, suggesting that the virus was fusing from an acidic compartment in which the fluorescence of AF was quenched. This was overcome by raising the endosomal pH with NH_4Cl. Thus, although the EBV fusion occurred within an intracellular compartment at low pH, the fusion was not dependent on acidic pH in order to occur.

CHAPTER 4
MODIFICATION OF THE ENDOCYTIC PATHWAY TO DETERMINE THE MECHANISM OF ACTION OF CHLOROQUINE ON VIRUS FUSION

Introduction

Since EBV enters B cells by endocytosis, one might assume that interference with this process would limit virus infectivity. This has been presumed to be at least partially responsible for the reduction in infectivity by calmodulin antagonists (Nemerow and Cooper, 1984b). It has been shown that inhibitors of oxidative phosphorylation and glycolysis can affect uptake of ligands into cells. Sodium azide, which inhibits oxidative phosphorylation, has been shown to inhibit partially the uptake of prebound Semliki Forest virus (Marsh and Helenius, 1980) and VSV (Blumenthal et al., 1987). It is possible that the inhibition of EBV fusion by chloroquine is due to a modification of the endocytic pathway that is inhibiting uptake of the virus into vesicles or altering the membrane so that virus is not able to fuse with the endosomal membrane. In addition to its pH-elevating property, chloroquine has other effects on lysosomal functions and other cellular processes (de Duve et al., 1974; Seglen, 1983). Chloroquine reduced uptake of asialo-fetuin (ASF) into cells when the concentration exceeded 0.1mM, and at concentrations above 1.0mM, chloroquine almost completely inhibited both uptake and degradation of ASF (Berg and Tolleshaug, 1980). Protease inhibition is another effect of chloroquine (Wibo, M. and B. Poole, 1974), in particular, inhibition of the enzyme cathepsin B (ibid) and phospholipases are also effected

(Matsuzawa and Hostetler, 1980). Chloroquine is also reported to alter membrane fluidity (Berg and Tolleshaug, 1980).

In order to investigate further the action of chloroquine on fusion of virus with B lymphocytes, fusion studies were done with cells treated with sodium azide, leupeptin, and chlorpromazine.

Materials and Methods

Membrane Fusion Assay

Epstein-Barr virus that had been labeled with the fluorophore octadecyl rhodamine B chloride (R_{18}) at self-quenching concentration was incubated with 2 X 10^6 cells and incubated for 1 hour on ice in the dark. When membrane fusion occurs there is dilution of the fluorophore in the membranes which relieves the self-quenching of the fluorescence. Cells were washed of unbound virus and the fluorescence emission was monitored continuously using a spectrofluorometer at an excitation wavelength of 560nm and an emission wavelength of 585nm. At the end of the assay Triton X-100 was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore.

Cells

The lymphoblastoid cell line Raji (Pulvertaft, 1964) and the recently EBV-transformed cell line BAT, which both express the virus receptor CR2, were grown at 37°C and diluted at least biweekly in RPMI 1640 supplemented with fetal calf serum and antibiotics. Fresh human T-depleted leukocytes were isolated from peripheral blood by flotation on LSM followed by rosetting with sheep erythrocytes and

centrifugation over 60% Percoll. The non-rosetting cells were collected from the gradient, extensively washed, and used directly in assays.

Preparation and Use of Cellular Inhibitors

Sodium azide, leupeptin, and chlorpromazine were purchased from Sigma (Sigma Chemical Co., St. Louis, Missouri). A 5mM stock solution of chlorpromazine was prepared in phosphate buffered saline (PBS) with 1.0% dimethylsulfoxide. Leupeptin was prepared by reconstituting 10mg of solid in 0.5ml distilled water to which 4.5ml of RPMI 1640 was added for a final concentration of 2mg/ml which was aliquoted and stored at -20°C. Stock solutions of the other inhibitors were prepared in PBS and all working dilutions were made in RPMI 1640. Cells were incubated with the inhibitors for 40 minutes at 37°, while control samples were incubated in RPMI medium alone. After incubation, cells were pelleted and the supernatant was removed and the cells were incubated with virus as described previously.

Results

Effect of Sodium Azide on Virus Fusion

Sodium azide (NaN_3), which inhibits oxidative phosphorylation, thus blocking the energy production required for endocytosis, had no effect on virus binding or fusion with Raji cells at a concentration of 10mM (Figure 4-1). When BAT cells were treated in the same manner, there was a 35% inhibition of fusion at 10mM (Figure 4-2), and some inhibition of binding was seen at 50mM. Virus fusion with peripheral T-depleted leukocytes was also partially inhibited with 10mM NaN_3 (Figure 4-3).

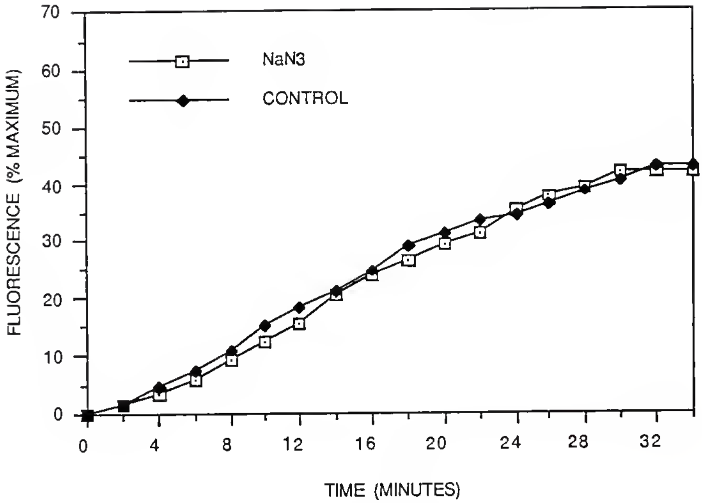


Figure 4–1. Effect of preincubation of Raji cells with sodium azide (NaN_3) or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

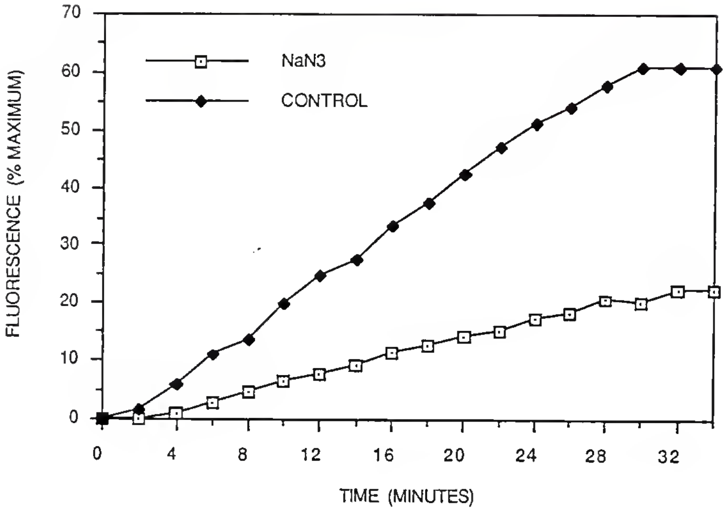


Figure 4-2. Effect of preincubation of BAT cells with sodium azide (NaN_3) or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

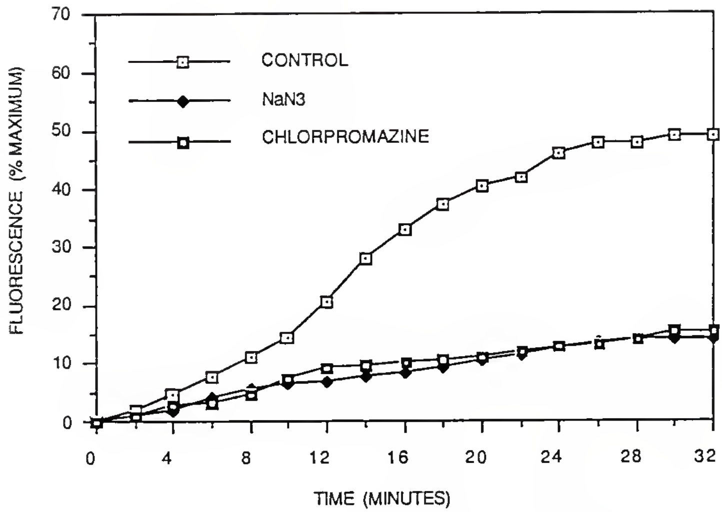


Figure 4-3. Effect of preincubation of T-depleted leukocytes with sodium azide (NaN_3), chlorpromazine, or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

Effect of Chlorpromazine on Virus Fusion

Chlorpromazine is a phenothiazine that acts as a calmodulin antagonist by binding to calmodulin. Phenothiazines have been shown to block EBV infectivity of human B cells (Nemerow and Cooper, 1984b). Chlorpromazine has been shown to exhibit this activity at a dose of 20uM with no impairment of virus binding. The endocytosis of immunoglobulin complexes, concavalin A and $alpha_2$-macroglobulin are inhibited by phenothiazines. The data in Figures 4-3, 4-4 and 4-5, show that 50uM chlorpromazine inhibited fusion with T-depleted leukocytes from 48.8% to 15.6% with no inhibition on virus binding, fusion with BAT cells was inhibited by 62%, but fusion with Raji cells was not inhibited.

Effect of Leupeptin on Virus Fusion

Leupeptin is N-propionyl- and N-acetylleucylleucyl-arginal in an approximately 3:1 ratio. This agent is a strong inhibitor of cysteine proteinases and inhibits the lysosomal cathepsins B, H, L, N, S, and T as well as the nonlysosomal Ca^{2+}-dependent proteinase II. It also inhibits a number of serine proteinases, including trypsin, plasmin, tissue kallikrein, and ribosomal serine proteinase. Leupeptin dissolves easily in aqueous solutions; for maximal effect it should be used at concentration of 100 ug/ml. Leupeptin has no effect on protein synthesis or ATP levels. The results in Figures 4-6, 4-7, and 4-8 show that this enzyme inhibitor had no detrimental effect on virus fusion with cells.

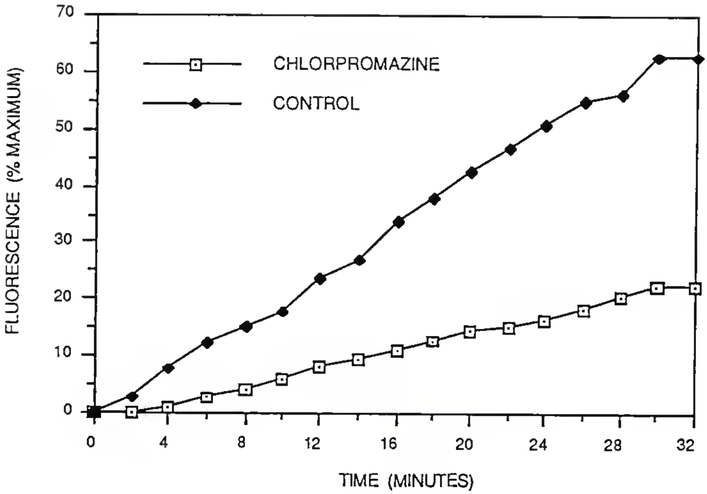


Figure 4-4. Effect of preincubation of BAT cells with chlorpromazine or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

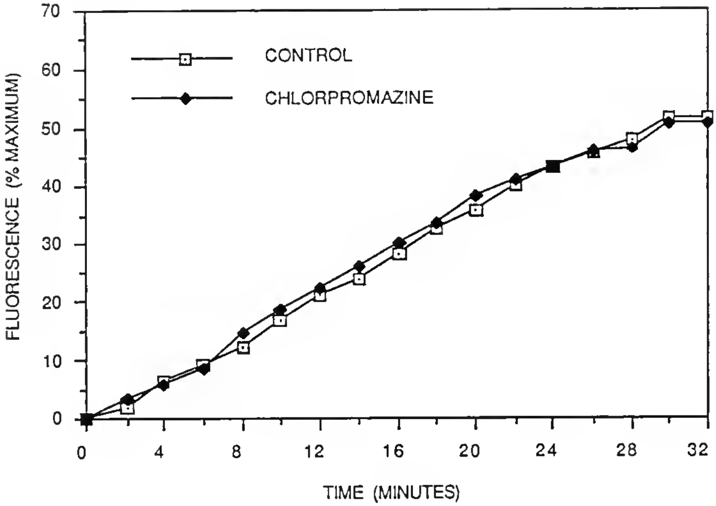


Figure 4–5. Effect of preincubation of Raji cells with chlorpromazine or RPMI on relief of self–quenching of R_{18}–labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X–100 (infinite dilution).

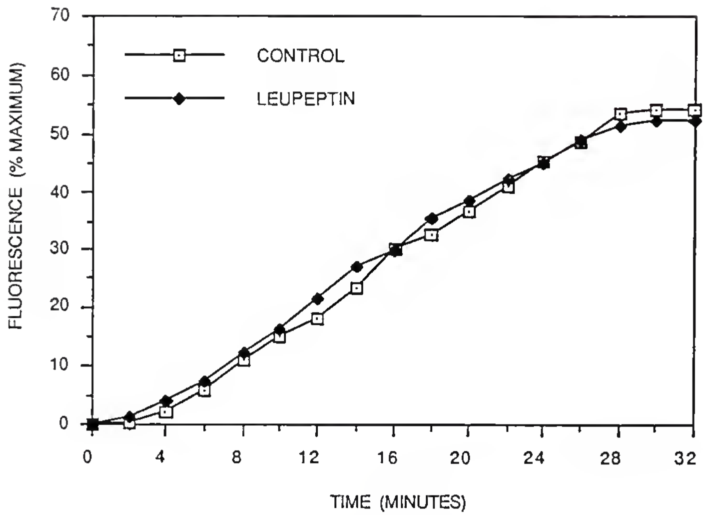


Figure 4-6. Effect of preincubation of Raji cells with leupeptin or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

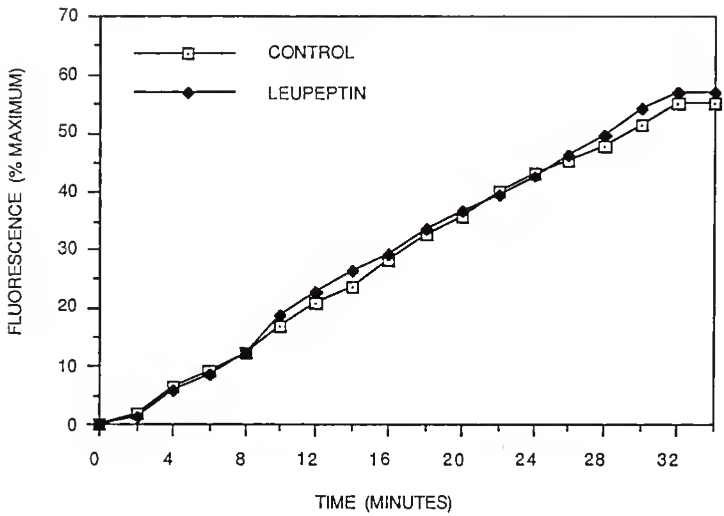


Figure 4-7. Effect of preincubation of BAT cells with leupeptin or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

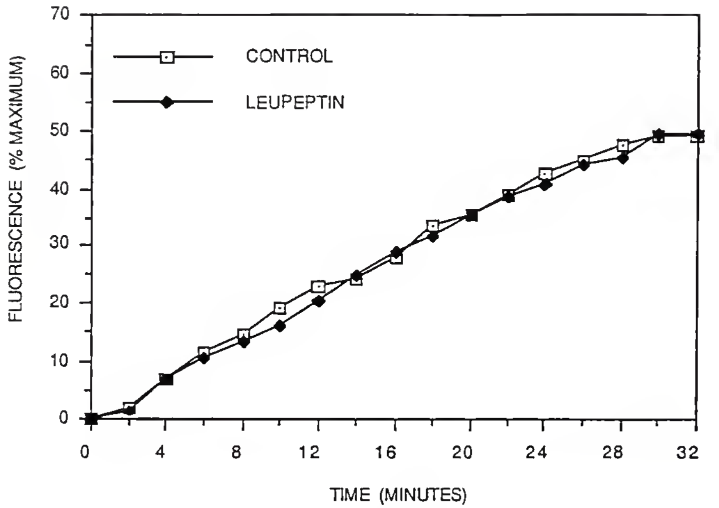


Figure 4–8. Effect of preincubation of T-depleted leukocytes with leupeptin or RPMI on relief of self-quenching of R_{18}–labeled MCUV5 virus. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

Discussion

The purpose of these studies was to elucidate the mechanism by which chloroquine inhibits virus fusion with fresh B lymphocytes and BAT cells, but not Raji cells. It had been established from the work presented in chapter 3 that this inhibition was not due to the effect that chloroquine has on the endosomal pH because other agents that elevate endosomal pH, such as methylamine and NH_4Cl, had no effect on fusion with any cell type. Two other possible effects of the drug were considered. First, since chloroquine only had effects on cells into which virus was endocytosed (see above and chapter 5) and the amount of dextran taken up into cells treated with chloroquine was less than that taken into untreated cells or cells treated with the other two lysosomotropic agents, it seemed possible that endocytosis was required for fusion with some cell types. Second, chloroquine has been reported to have activity as an inhibitor of cathepsin B and also some phospholipases (Wibo and Poole, 1974). Proteolytic cleavage is a prerequisite for function of several viral fusion proteins (White et al, 1983). Although this process usually occurs during maturation of virus it is possible that activation of fusion via proteolysis could occur following binding of virus to the host cell (Hattori et al., 1989) and be required for fusion with the normal B cell. The first of these possibilities was examined by testing the effects of two other inhibitors of endocytosis on virus fusion.

Sodium azide (NaN_3) inhibits oxidative phosphorylation in cells which affects their endocytic capacity; fusion of VSV via the endocytic pathway was inhibited by treatment of the cells with NaN_3 (Blumenthal et al., 1987). Fusion of EBV with Raji cells was not inhibited by sodium azide, which would be expected because the virus fuses at the plasma membrane, but both BAT cells and T-depleted leukocytes treated

with the agent were inhibited in their ability to permit virus membrane fusion. The phenothiazine drug, chlorpromazine, has also been reported to inhibit endocytosis of some ligands although by a different mechanism than NaN_3 (Salisbury et al., 1980, 1981; Cheung et al., 1983). Chlorpromazine binds to calmodulin, which is a calcium-binding protein that has multifaceted involvement in regulation of cellular processes (Cheung, 1980). Chlorpromazine exerts its activity by blocking the stimulation of Ca^{2+}-dependent phosphodiesterase by calmodulin. This drug inhibited fusion of virus with BAT cells by 40% and fusion of virus with T-depleted leukocytes was decreased from 48.8% to 15.6%. Raji cells were marginally effected by treatment with this drug. Chlorpromazine was also shown to block virus infectivity of human B cells as measured by outgrowth of transformed cell colonies or by stimulation of DNA synthesis (Nemerow and Cooper, 1984b).

The second possibility was tested with the protease inhibitor leupeptin which has potent activity for inhibiting cathepsin B along with possessing protease inhibiting activities for many other enzymes. Leupeptin failed to inhibit virus fusion with any cell types.

These experiments strongly suggest that inhibition of endocytosis in BAT cells and normal B cells compromises their ability to fuse with EBV. It is not clear why virus should be able to fuse efficiently at the plasmalemma of Raji cells and apparently not at the plasmalemma of normal B cells but this perhaps reflects differences in the composition of the membranes of these different cells. Fusion of viruses with any membrane is dependent on many factors including the lipid composition of the membrane (Bramhall and Wisnieski, 1981). Altering the lipid composition of the Raji cell membrane has been shown to inhibit virus fusion (Patel, Hutt-Fletcher, and Crews;

personal communication) and it is reported that the endosomal membrane has a different lipid composition from that of the plasmalemma. One possibility that will require further examination is that the lipid composition of the normal B cell plasmalemma (but not the endosome) is different from that of the Raji cell and is thus not capable of fusing with the envelope of EBV.

CHAPTER 5
ISOLATION AND IDENTIFICATION OF EPITHELIAL CELLS EXPRESSING A RECEPTOR FOR EPSTEIN-BARR VIRUS AND STUDIES OF VIRUS ENTRY INTO THESE CELLS

Introduction

The ability of EBV to infect lymphocytes is initiated by attachment of virus to the cell membrane glycoprotein CR2, which also binds the C3d fragment of complement (Fingeroth et al., 1984; Nemerow et al., 1985b). CR2 is a 145-kilodalton B cell membrane glycoprotein (Weis et al., 1984) whose precise function is not known, but appears to be immunoregulatory (Cooper et al., 1988). The B cell CR2 is lost with activation and differentiation to the plasma cell. CR2 has been assigned to the CD21 antigen cluster of B cell differentiation (Reinherz et al., 1986).

Epithelial cells have been shown to express a receptor for virus attachment in a differentiation-dependent manner. The receptor has been detected on the surface of non-keratinized squamous cells and deeper layers (Sixbey et al., 1987; Young et al., 1986). The receptor expression appears limited to the less differentiated cells, although there is some confusion in the literature about its precise distribution. Epithelia in certain anatomical sites can support EBV infection, most dramatically demonstrated in oral hairy leucoplakia and nasopharyngeal carcinoma, but the functional significance of a CR2-like molecule on epithelial cells remains yet undetermined. Epithelial involvement in both acute infection and the chronic carrier

state is consistent with the concept that the virus persists in part via low grade replication in the oropharynx.

It is well accepted that virus infects epithelial cells, but the question of how the virus gains access to epithelial cells remained unresolved. The membrane fusion assay provides an opportunity for addressing this important aspect of EBV infection. The purpose of the work presented in this chapter was to extend studies of virus entry into lymphocytes to include epithelial cells.

Materials and Methods

Isolation of Epidermal Cells from Foreskin Tissue

Foreskin tissue from newborns was collected after surgery and placed in DMEM (Sigma Chemical Co., St. Louis, Missouri) with 10% fetal calf serum and antibiotics. The tissue was rinsed with PBS and subcutaneous fat was removed using a small iris scissors. The tissue was cut into 3-5 mm^2 pieces and incubated for 9-12 hours at 4°C in 25ml F-12 media containing dispase (Sigma) at 0.75 units/ml (Stenn et al., 1989). After enzymatic treatment, the epidermis was easily peeled away from the dermis using a scalpel and forceps. The pieces of epidermis were incubated with 15-20ml of 0.1% trypsin with EDTA (Sigma) for 35-40 minutes at 37°C with stirring. Epidermal cells were collected in the supernatant after inactivation of trypsin with serum. Cells were washed by centrifugation at 1500 r.p.m., resuspended in 4ml of DMEM (Sigma) and layered onto a discontinuous 30 (3ml), 40 (3ml), 45 (3ml), and 100% (1ml) isotonic Percoll gradient. The gradient was centrifuged for 30 minutes at 1500 r.p.m. at 4°C. Cells banded at the media/30%, 30/40%, 40/45% and the

45/100% interfaces. Each of these populations of cells was collected separately and extensively washed before use in experiments.

Cell Lines

The human B lymphoblastoid cell line Raji was grown at 37°C and diluted at least biweekly in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin and 100ug of streptomycin per ml.

Virus Production

Virus-producing cells were induced with 30ng of 12-O-tetradecanoylphorbol-13-acetate per ml, and after 7 days, virus was collected from the spent culture medium. The cells were centrifuged at 4,000 X g for 10 minutes to remove cells; 100ug of bacitracin per ml was added to the clarified supernatant, and the virus was pelleted by centrifugation at 20,000 X g for 90 minutes. Pellets were suspended in 1/250 of the original volume of medium containing 100ug of bacitracin per ml, reclarified by centrifugation three to four times at 400 X g, and filtered through a 0.45um pore filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.).

Incorporation of R_{18} and AF into Virus Membranes

A stock solution of 13nmol of R_{18} per ul (Molecular Probes, Inc., Junction City, Oregon) was prepared in chloroform-methanol (1:1,v/v) and stored at -20°C. The probe was incorporated into virus membranes by a modification of the method of Hoekstra and colleagues (Hoekstra et al., 1984). Three microliters of the stock probe were dried under nitrogen and solubilized in ethanol, and 15ul of this solution containing 15nmol of R_{18} was added to 250ul of virus with vortexing. The probe and virus were incubated at room temperature in the dark for 1 hour, after which the virus and unincorporated R_{18} were separated by chromatography on Sephadex G-75

(Sigma). Labeled virus was stored at -70°C. Labeled virus was still infectious and retained binding specificity of unlabeled virus.

A stock solution of 50mg/ml of AF (Molecular Probes, Inc., Junction City, Oregon) was prepared in dimethylformamide and stored at -20°C. The probe was incorporated into the virus membrane and stored in the same manner as the R_{18} probe.

Fluorescence Measurement

R_{18} labeled virus (or AF-labeled virus) was added to pellets of 2 X 10^8 cells and incubated for 1 hour on ice in the dark. Cells were washed four times with ice-cold Dulbecco's saline, suspended in 400ul, and transferred to the microcuvette of the spectrofluorometer (SLM SPF500C) equipped with a magnetic stirrer and circulating water bath. For the R_{18}-labeled virus, fluorescence was monitored continuously at an excitation wavelength of 560nm and an emission wavelength of 585nm, AF-labeled virus was measured at an excitation wavelength of 496nm and an emission wavelength of 522nm. At the end of the assay, Triton X-100 (1% v/v, final concentration) was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore.

Cells were also analyzed for virus binding and fusion by direct fluorescence microscopy with R_{18}-labeled virus and evaluated for receptor expression by indirect immunofluorescence microscopy. Cells were incubated with R_{18}-labeled virus for 35 minutes at 37°C, washed of excess virus and mounted onto a slide for analysis. For indirect immunofluorescence, cells were incubated with monoclonal antibody, washed three times and incubated with FITC-conjugated sheep anti-mouse immunoglobulin G and washed again before analysis.

Monoclonal Antibodies

Several antibodies that react with CR2 were used: OKB7, which blocks virus binding to B cells and HB5, B2, 2G7, 6F7, 1F8, 1C8, and LO2 which do not. The antibodies OKB7, 2G7, 6F7, 1F8, 1C8, and LO2 were gifts from Barry Myones (Children's Memorial Hospital, Chicago, Illinois) and the antibody B2 was a gift from Stephen Pflugfelder (Bascom Palmer Eye Institute, University of Miami, Miami, Florida). The HB5 hybridoma cell line was obtained from the American Tissue Culture Collection (Rockville, Maryland) and the antibody was purified from culture medium by immunoaffinity chromatography on protein A sepharose (Genzyme).

Fluorescence-Activated Cell Sorter Analysis (FACS)

Epithelial cells for FACS analysis were isolated from tissue and separated on Percoll as previously described and the cells at the 45/100% interface were incubated with HB5 antibody for 35 minutes at 37°C followed by three washes. Cells were then incubated with FITC-conjugated-sheep anti-mouse IgG for 35 minutes at 37°C followed by three washes. Control cells received the second antibody only. Samples of 1×10^6 control cells and 2×10^6 test cells were analyzed to determine the level of fluorescence in order to position the gating for sorting and collection of the positive and negative cells separately. The cells to be sorted were at a concentration of 3×10^6 per ml. Analysis was performed using the FACStar plus (Becton-Dickinson).

Preparation and Use of Lysosomotropic Agents and Sodium Azide

Cells were incubated for 35 minutes at 37°C with the lysosomotropic agents ammonium chloride (NH_4Cl), chloroquine and methylamine, followed by incubation with virus. Sodium azide, which inhibits oxidative phosphorylation was incubated with cells for 35-40 minutes at 37°C followed by incubation with virus.

Results

Isolation of Single Cells From Foreskin Tissue

The sequential digestion of the foreskin tissue with dispase and trypsin allowed disaggregation of the tissue and release of individual cells. This treatment did not result in complete digestion of the pieces of epidermis into single cells, but the cells that were released from the tissue were healthy in terms of viability as determined by exclusion of the dye trypan blue (Table 5-1). The mean value for the number of cells isolated from tissue was 1.3×10^7 with a standard deviation of 4.7×10^6 cells. The difference in the number of cells released from tissues in Table 5-1 primarily reflected differences in the starting surface area of the tissue. The cells were separated by size on discontinuous Percoll gradients in order to obtain populations of cells that would reflect the normal differentiation process of the cells in the epidermis. The epidermis consists of multiple layers of epithelial cells, called keratinocytes. Mitosis is restricted to the basal layer and keratinocytes that leave this layer undergo terminal differentiation as they move upwards towards the tissue surface. Typically, as the cells differentiate, they increase in size and the shape of the cells changes from small and rounded to enlarged, irregularly shaped cells. The cells obtained from the interfaces of the Percoll gradient were evaluated separately on a morphological basis into three categories, basal, parabasal and squamous cells and the results of this analysis are presented in Table 5-2. The basal cells are the smallest cells and they are the proliferative cells of the epidermal layer. These cells are found primarily at the 45/100% interface of the gradient, although a small percentage of these cells are found throughout the gradient, most likely due to trapping by the larger cells. Parabasal cells have a larger cytoplasm than the basal cells, but are distinguishable

Table 5-1. Cell counts and viability of cells recovered from infant foreskin epidermis.

number of cells	viability[a]
4.95×10^6	89%
1.84×10^7	93%
2.17×10^7	94%
1.90×10^7	86%
1.14×10^7	96%
1.22×10^7	98%
1.29×10^7	95%
1.54×10^7	93%
9.10×10^6	96%
1.41×10^7	95%
1.26×10^7	87%
7.42×10^6	87%
1.02×10^7	99%
1.74×10^7	95%
8.6×10^6	95%

[a]determined by trypan blue exclusion

Table 5-2. Morphological distribution of epithelial cells in fractions from Percoll gradient.

gradient[a] fraction	% of total	distribution of cells (%)		
		basal	parabasal	squamous
1-media/30% interface	9.2 ±2.3	6.2 ±1.2	18.4 ±3.5	75.4 ±6.2
2-30/40% interface	15.8 ±8.1	17.3 ±3.4	66.2 ±7.9	16.5 ±5.9
3-40/45% interface	26.5 ±5.2	23.5 ±5.6	71.5 ±6.9	5.0 ±3.0
4-45/100% interface	47.9 ±10.3	71.2 ±7.6	28.8 ±7.4	0

[a]30%. 40%, 45%, 100% Percoll

from the very large keratinized squamous cells. Parabasal cells were found at both the 45/45% and the 35/40% interfaces. There is no significant morphological difference between cells seen at these two interfaces except that more basal cells were found at the 40/45% interface. The migration of the parabasal cells into two fractions may reflect differences in the extent of the keratinization of these cells. The two fractions were handled as two different populations when evaluating virus binding and receptor expression. The terminally differentiated squamous cells were found at the media/30% interface. These cells were very large in comparison to the other cell types and a majority of the non-viable cells in the total population were of this type.

Reactivity of Epithelial Cells with Anti-CR2 Antibodies

Epithelial cells that were separated by size on discontinuous Percoll gradients into four populations were analyzed for expression of a CR2-like molecule using the anti-CR2 antibody HB5 (Weis et al., 1984). Expression of this receptor has been demonstrated on cells separated in this manner from cultures epithelial cell explants (Sixbey et al., 1987). Table 5-3 illustrates the results of this experiment. The large keratinized squamous cells which were found at the media/30% interface exhibited very little staining with this antibody. Cells that were found at the 30/40% and the 40/45% interfaces exhibited the highest amount of reactivity. The small basal cells found at the 45/100% interface exhibited more staining than the squamous cells but not as much as the suprabasal cells.

On subsequent analysis only parabasal and basal cells were analyzed due to lack of reactivity of squamous cells with HB5 and also because it was more likely that the less keratinized cells would actually bind and internalize virus. Table 5-4 illustrates the results of binding assays of epithelial cells with additional anti-CR2

Table 5-3. Reactivity of epithelial cells with the monoclonal anti-CR2 antibody HB5.

gradient fraction	percentage positive cells
1-media/30% interface	5 ± 3
2-30/40% interface	29 ± 8
3-40/45% interface	21 ± 6
4-45/100% interface	6 ± 3

Table 5-4. Reactivity of epithelial cells with anti-CR2 monoclonal antibodies.

Monoclonal antibody	% positive cells	
	parabasal	basal
HB5[a]	27	8
B2	66	45
OKB7	0	0
2G7	0	0
1F8	0	0
1C8	0	0
LO2	0	0

[a]Antibody binding was visualized with FITC-labeled anti-mouse antibody.

antibodies. No cells stained detectably with the antibody OKB7 which is known to be able to inhibit virus binding to B cells (Nemerow et al., 1985a) although the epitope recognized by this antibody has been mapped very close to the EBV binding site and the binding site of the natural ligand, C3d (Lowell et al., 1989). Of the other anti-CR2 antibodies that were used, only two, HB5 and B2 consistently gave positive staining. These data indicate that there are parabasal and basal cells that express a molecule that reacts with anti-CR2 antibodies and that the amount of this reactivity increases from the basal to parabasal phenotype as judged by size of the cells.

Virus Binding and Fusion with Epithelial Cells by Microscopic Analysis

Virus binding and fusion was measured by incubating cells with R_{18}-labeled EBV and looking for rhodamine stained cells under the fluorescence microscope. Cells were also fixed with 0.1% paraformaldehyde and assayed in parallel; fixing with paraformaldehyde has been shown to prevent virus fusion as measured by relief of self-quenching of R_{18}-labeled EBV bound to B cells. The data in Table 5-5 show that 20% of the parabasal cells and 26% of basal cells were positive for R_{18} staining and this was reduced to 1% and 3% respectively by fixation of the cells, confirming that unfused virus was labeled with quenched amounts of R_{18}. Cells were also stained in parallel with the monoclonal antibody HB5, with which 27% of the parabasal cells and 8% of the basal cells were positive. A larger percentage of basal cells stained with labeled virus than were identified as having a CR2-like molecule as determined by reactivity with the HB5 antibody. Fixation of cells had little effect on parallel staining done with HB5, 24% of parabasal and 7% of basal cells were positive, these results assured that lower values obtained for HB5 staining of basal cells could not be attributed to internalization of receptor by antibody.

Table 5-5. Microscopic analysis of virus binding and fusion with epithelial cells.

Treatment	parabasal[a]		basal[a]	
	unfixed	fixed	unfixed	fixed
EBV-R_{18}	20±5	1±2	26±6	3±1
HB5[b]	27±7	24±3	8±4	7±5

[a]Results expressed as percent positive staining cells.
[b]Antibody binding was visualized with FITC-labeled anti-mouse antibody.

Relative Kinetics of Virus Fusion by Spectrofluorometric Analysis

The kinetics of relief of self-quenching of virus bound to parabasal and basal cells was analyzed on a population basis in the spectrofluorometer. Figure 5-1 shows the fluorescence dequenching curves of parabasal and basal cells which were essentially identical for the two cell populations. Approximately 50-60% of the bound R_{18}-labeled virus was dequenched within 35 minutes. These values are comparable to those that we have previously obtained with lymphocytes. The amount of labeled virus that was bound to cells could be evaluated by comparing the values for maximum fluorescence upon addition of Triton X-100 to cells and virus. These values were about twice as high for the basal cells as for parabasal cells despite the fact that fewer of the basal cells stained with HB5 or B2 antibody. In comparison to a population of Raji cells, in which greater than 90 per cent of the cells stain with HB5 antibody, the amount of virus binding to basal cells, on a per cell basis for cells reacting with HB5, is two to three times higher for the basal cells than the Raji cells.

FACS Analysis of Basal Epithelial Cells

The cell sorting capability of a flow cytometer makes it possible to isolate relatively pure populations of cells with defined characteristics. This technique of cell sorting first requires that the stream containing the sample of cells is broken into droplets that are a fixed distance from the observation point so that individual cells can be analyzed. Between the time a cell transverses the observation point and the time it reaches the droplet breakoff point, the cell is analyzed for fluorescence and based on the signal level that has been determined as positive or negative from an analysis of a sample of the population to be sorted. As each droplet breaks off from the stream it is electrostatically charged if it is to be collected. Charged droplets are

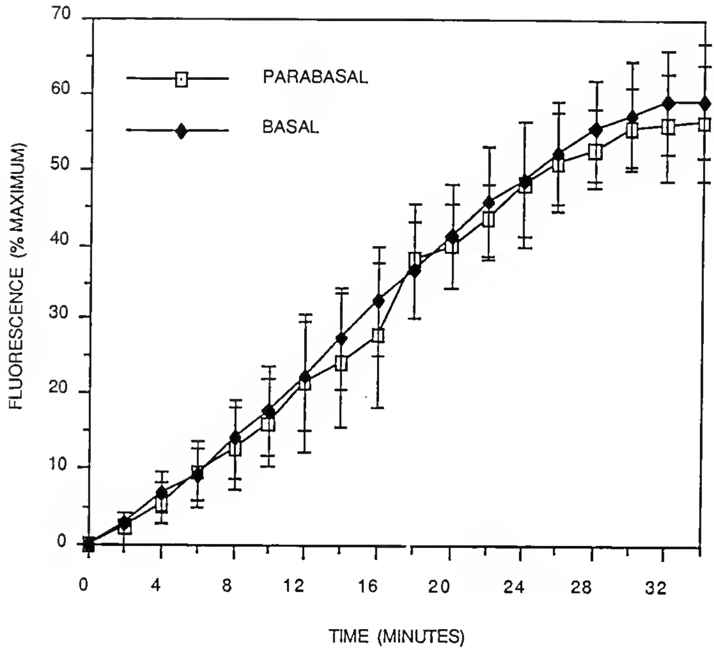


Figure 5-1. Relief of self-quenching of R_{18}-labeled MCUV5 virus bound to parabasal and basal epithelial cells. Increase in fluorescence is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

deflected out of the main stream toward the deflection plate bearing an opposite charge, and the charged droplet streams are collected, while the uncharged main stream passes into an aspiration tube leading to the waste reservoir. The sorter also permits formation of two sort streams, one positively charged and the other negatively charged, which allows isolation of cells selected by two criteria (Shapiro, 1988).

To examine the virus receptor on the epithelial basal cell population further, HB5 positive and negative cells were sorted in the fluorescence activated cell sorter. FACS analysis seemed to be considerably more sensitive than our previous microscopic analysis and indicated that approximately 19% of basal cells were HB5 positive (Figure 5-2). The HB5 negative population was recovered from the population and then analyzed for its ability to bind and to fuse with R_{18}-labeled EBV. Figure 5-3 shows a spectrofluorometric analysis of virus bound to unsorted basal cells and two sorts of basal cells from which the HB5 reactive population was removed. Removal of the HB5 positive population reduced the amounts of virus binding by 85 to 90%. The numbers of HB5 positive cells recovered were too small for analysis in the spectrofluorometer, but microscopic analysis of these cells indicated that greater than 85% of these cells were able to bind and fuse with virus.

Effects of Lysosomotropic Agents on Virus Fusion

Lysosomotropic agents cross cell membranes easily in the unprotonated form but the protonated form does this far less efficiently. When the uncharged form enters acidic vesicles it become protonated, thereby raising the pH and inhibiting its own escape across the membranes of the vacuoles. Basal epithelial cells were treated with the lysosomotropic agents chloroquine, ammonium chloride (NH_4Cl), and methylamine and evaluated for the ability to bind and fuse R_{18}-labeled virus. These

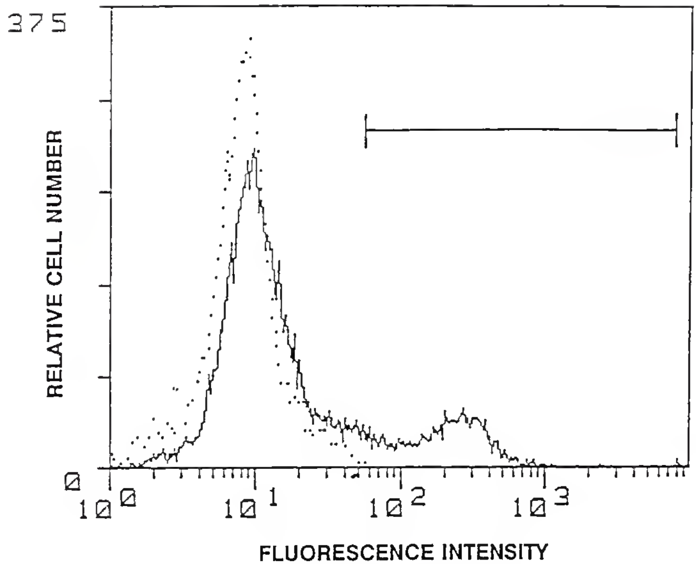


Figure 5-2. Fluorescence profile of HB5 antibody binding to basal epithelial cells. Antibody was visualized with FITC-conjugated anti-mouse antibody. Broken line is control profile of cells incubated with FITC-mouse anti-immunoglobulin alone. The horizontal line indicates the portion of the gate used to sort cells into HB5(+) and HB5(-) populations. The proportion of cells included in this gate was 18.99% of the total.

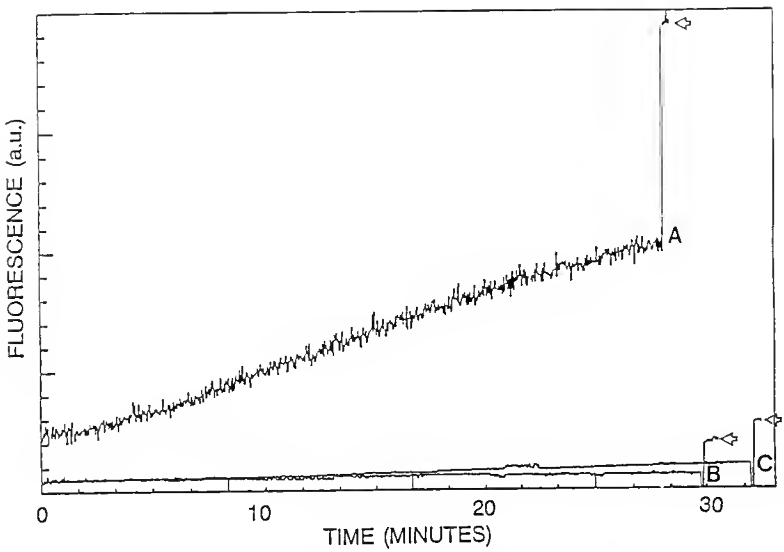


Figure 5-3. Relief of self-quenching of R_{18}-labeled virus bound to unsorted basal epithelial cells (A) or basal epithelial cells from which HB5(+) cells were removed by cell sorting (B and C). Fluorescence expressed in arbitrary units (a.u.). Arrow indicates point at which Triton X-100 was added to measure maximum relief of self-quenching of bound virus (infinite dilution).

agents have been widely used to determine if virus fusion is dependent on low pH in order to occur. They were used to determine that fusion of EBV with lymphocytes is not catalyzed by low pH. Figures 5-4 and 5-5 show that chloroquine, NH_4Cl, and methylamine did not have any inhibitory effects on virus fusion with basal epithelial cells.

Effects of Sodium Azide on Virus Fusion

Sodium azide, which inhibits endocytosis in cells, was able to inhibit virus fusion with B lymphocytes, but not with the lymphoblastoid cell line Raji. Basal epithelial cells were treated with 10mM sodium azide followed by incubation with virus. Sodium azide had no effect on virus fusion with epithelial cells (Figure 5-6).

Fusion of AF-labeled Virus

Virus labeled with AF has proven to be a valuable method for determining whether virus fusion is occurring in a neutral or acidic environment from previous experiments with lymphoblastoid cell lines and fresh B cells. AF-labeled virus was incubated with cells that had been treated with NH_4Cl to neutralize acidic compartments and untreated cells. The results in Figure 5-7 show that fluorescence dequenching could be measured with the AF-labeled virus regardless of whether or not the cells were treated with NH_4Cl.

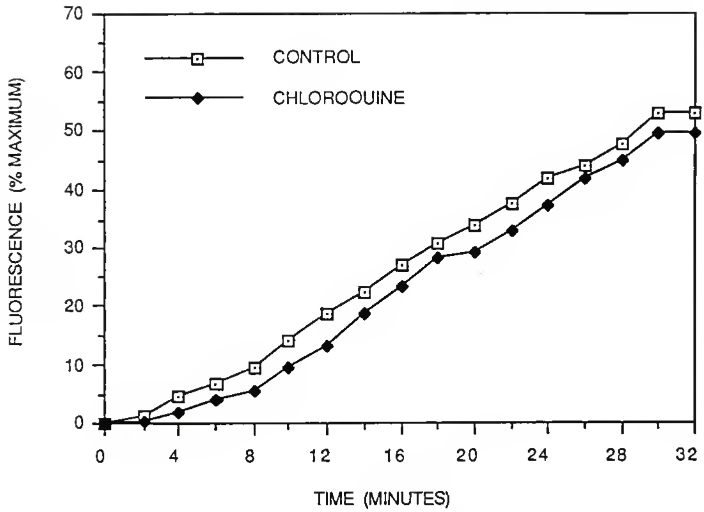


Figure 5-4. Effect of preincubation of basal cells with chloroquine or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence expressed as a percent of the maximum release after addition of Triton X-100 (infinite dilution).

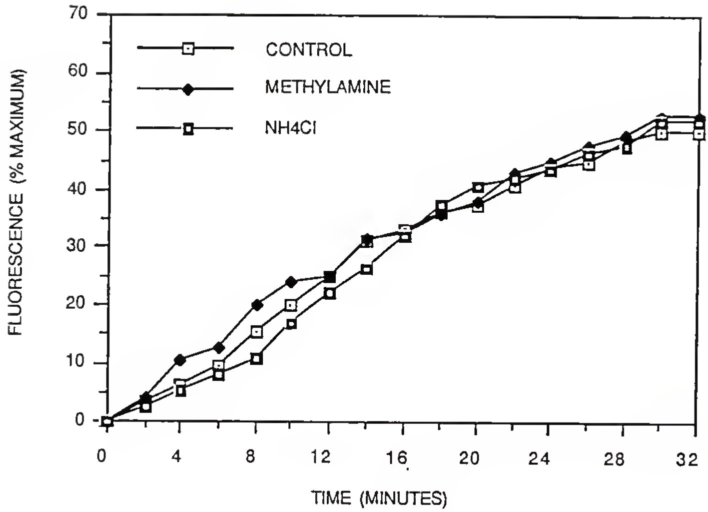


Figure 5-5. Effect of preincubation of basal cells with methylamine, NH_4Cl, or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence expressed as a percent of the maximum release after addition of Triton X-100 (infinite dilution).

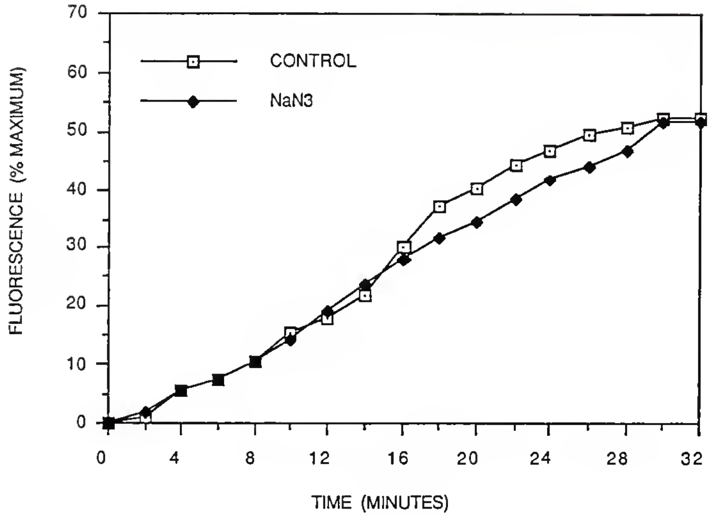


Figure 5-6. Effect of preincubation of basal cells with sodium azide (NaN_3) or RPMI on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to cells. Increase in fluorescence expressed as a percent of the maximum release after addition of Triton X-100 (infinite dilution).

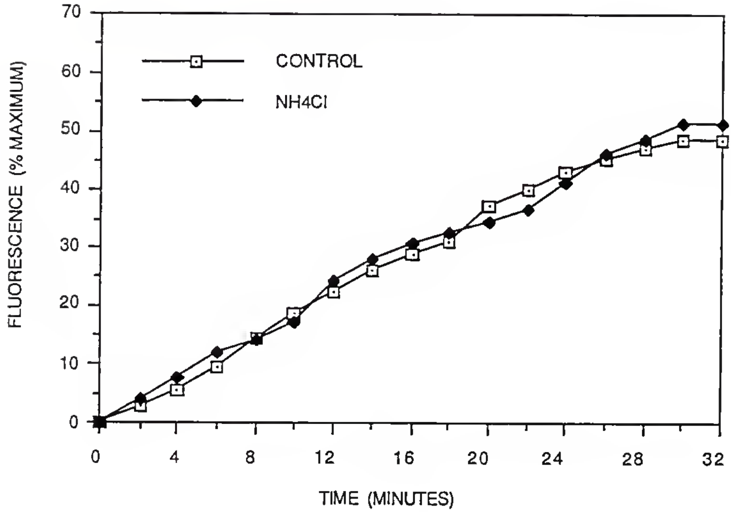


Figure 5-7. Effect of preincubation of basal cells with NH_4Cl or RPMI on relief of self-quenching of AF-labeled MCUV5 virus bound to cells. Increase in fluorescence expressed as a percent of the maximum release after addition of Triton X-100 (infinite dilution).

Discussion

Epstein-Barr virus can be found in saliva in most patients with infectious mononucleosis and at low levels in almost all healthy EBV seropositive persons (Chang et al., 1973; Golden et al., 1973; Miller et al., 1973; Niederman et al., 1976; Yao et al., 1985). The ease with which EBV is recovered from oral secretions of persons with primary or reactivated EBV infections suggests that a cell type freely permissive of EBV replication exists in the oropharynx (Morgan et al., 1979). Increasing evidence has suggested that EBV can infect and replicate in epithelial cells (Greenspan et al., 1985; Lemon et al., 1977; Sixbey et al., 1983, 1984, 1987). Even though is it well accepted that EBV infects two lineages of cells, B lymphocytes and epithelial cells, the question of how EBV gains access to epithelial cells is still not resolved.

Initially, the anti-CR2 antibody HB5 was used to determine which cells from the epidermal tissue were expressing a receptor for virus. This antibody has been used by other investigators to locate CR2-positive cells in epithelium from various areas of the body (Sixbey et al., 1987; Thomas and Crawford, 1989; Young et al., 1986, 1989). By using this reagent it was determined that the highest amount of reactivity was located in the parabasal cell population. Many other antibodies that detect the CR2 molecule on B cells were also tested for their ability to detect this molecule on epithelial cells. Of the other antibodies tested, only one, B2 (Nadler et al., 1981; Iida et al., 1983) reacted with epithelial cells. However, the B cell CR2 molecule is composed of 15 or 16 repetitive elements and is a member of a gene family which has a high degree of polymorphism (Fujisaku et al., 1989; Toothtaker et al., 1989). It is then perhaps not surprising that the epithelial CR2-related molecule might not be

recognized by all the monoclonal reagents selected for reactivity with the B cell CR2. Polymorphic variations in the CR2 coding sequence, resulting both from alternative splicing of exons in the DNA and from the existence of allelic differences could result in generation of distinct forms of the protein in different cell types. More surprising, however, was the finding that the monoclonal antibody OKB7, which inhibits EBV binding with the B cell, (Carel et al., 1990; Nemerow et al., 1985a) did not react with the epithelial cells which bound virus. OKB7 has been mapped to the terminal SCR at a site very close to, though not identical to the viral binding site (Lowell et al., 1989).

The goal of this work was to investigate virus binding and entry into epithelial cells in order to compare it to lymphocytes. When cells from the basal cell population and the parabasal cell population were evaluated for their ability to bind and internalize virus as measured by the fluorescence dequenching assay, there was not a strict correlation between virus binding and reactivity of the cells with the HB5 antibody. Cells from the basal cell population bound 50 per cent more virus than cells from the parabasal population despite the lack of reactivity of basal cells with the HB5 antibody. The amount of fluorescence dequenching of the virus bound to the two populations was similar, reflecting an equal ability of functional virus to enter these cells.

The basal cell population represents approximately 50 per cent of the total number of cells isolated from the epidermal pieces of tissue and this population was able to bind and internalize virus. The entry of virus into these cells was pursued further in studies utilizing lysosomotropic agents that raise the intracellular pH. The three agents tested in this work, chloroquine, NH_4Cl, and methylamine had no inhibitory effects on virus binding and fusion with basal epithelial cells. These results

indicate that virus fusion was not dependent on low pH in order to occur and this parallels what has been found with the lymphoblastoid cell line Raji. Virus entered Raji cells by fusion at the plasma membrane, in contrast with freshly isolated T-depleted leukocytes, in which virus was endocytosed and virus fused from within an endocytic vesicle. T-depleted leukocytes were not inhibited by methylamine or NH_4Cl, but virus fusion with chloroquine treated cells was inhibited possibly due to reduced endocytosis of these cells after chloroquine exposure. Sodium azide, which also inhibits endocytosis because of its effects on oxidative phosphorylation of the cell, was able to inhibit virus fusion with T-depleted leukocytes, but did not inhibit fusion of virus with basal epithelial cells. Further confirmation that virus fusion occurs at the plasma membrane of basal epithelial cells was obtained from fusion assays in which fluorescence dequenching of virus labeled with R_{18} was compared with that of virus labeled with the pH sensitive probe AF. The dequenching curves were superimposable, indicating that virus is not exposed to a low pH environment before it fuses with the epithelial cell.

Further work will be necessary to understand why the level of virus binding to basal cells does not parallel the amount of receptor detectable with known anti-CR2 reagents.

CHAPTER 6
EFFECTS OF MONOCLONAL ANTIBODIES TO VIRUS MEMBRANE PROTEINS ON BINDING AND ENTRY OF EPSTEIN-BARR VIRUS INTO LYMPHOCYTES AND EPITHELIAL CELLS

Introduction

Previous studies on the function of EBV membrane glycoproteins have focused on the largest and most abundant molecules, gp350 and gp220, primarily because antibodies that recognized these proteins were capable of neutralizing virus infectivity by inhibiting virus from binding. Even though the majority of neutralizing activity in normal sera can be accounted for by antibody that reacts with these molecules (Thorley-Lawson and Poodry, 1982), the predominant anti-membrane reactivity in neutralizing sera taken from patients during the acute phase of infectious mononucleosis is directed against another envelope protein, gp85 (Qualtiere and Pearson, 1979). The development of an assay to measure virus entry by measuring membrane fusion was pursued in the interest of investigating the function of viral membrane proteins in events occurring after virus binding.

Monoclonal antibodies are a valuable tool for identifying proteins and their specificity has been widely used for virus function studies in many systems. The studies presented in this chapter were in pursuit of greater understanding of which viral surface membrane proteins are important for virus fusion.

Materials and Methods

Cells

The lymphoblastoid cell lines used, Raji and BAT, were grown at 37°C and diluted at least biweekly in RPMI 1640 (Sigma Chemical Co., St. Louis, Missouri) supplemented with heat-inactivated fetal calf serum, 100 IU of penicillin and 100ug of streptomycin per ml. Both cell lines are latently infected with EBV and express the virus receptor CR2.

Fresh human peripheral leukocytes were obtained by flotation on LSM followed by depletion of T lymphocytes by rosetting with sheep erythrocytes. These cells were used in assays directly after isolation.

Human epithelial cells were isolated from foreskin tissue by a sequential enzymatic digestion with dispase (Stenn et al., 1989) to separate the epidermis from the dermis, followed by digestion of the epidermal sheets with trypsin. The suspension of epidermal cells were separated further by centrifugation on a 30, 40, 45, 100% discontinuous Percoll gradient. Cells from the 40/45% interface and the 45/100% interface were collected and used as two separate population of cells, respectively, parabasal and basal cells.

Monoclonal Antibodies

Monoclonal antibodies were purified from hybridoma culture supernatants by chromatography on protein A-Sepharose. Five anti-viral antibodies were studied, two of the antibodies recognize gp85; F-2-1 (Strnad et al., 1982), of the IgG2a subclass, and E1D1 (Balachandran et al., 1987), of the IgG1 subclass. E8D2 (Balachandran et al., 1986) is an IgG2a antibody that recognizes the EBV-induced early membrane

protein p105. An antibody that recognizes the major envelope glycoprotein gp350/220, 72A1 (Hoffman et al., 1980) was also used.

Membrane Fusion Assay

Epstein-Barr virus that has been labeled with R_{18} at self-quenching concentration was added to 2 X 10^6 cells and incubated for 1 hour on ice. Cells were washed four times with ice cold Dulbecco's saline and suspended in 400ul of Dulbecco's when transferred to the microcuvette of a spectrofluorometer. Fluorescence dequenching was monitored continuously at an excitation wavelength of 560nm and an emission wavelength of 585nm. At the end of the assay, Triton X-100 (1% v/v, final concentration) was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore.

Soluble CR2

Binding inhibition assays were done using a soluble form of the first two short consensus repeats (SCR) of the CR2 protein. This reagent was a gift from Glen Nemerow (Research Institute of Scripps Clinic, La Jolla, California). Raji cells and basal epithelial cells from newborn foreskin tissue were evaluated for their ability to bind R_{18}-labeled EBV in the presence of soluble CR2. Virus that had been preincubated with the anti-viral antibody 72A1 was also included in the experiments. Cells were either incubated with 72A1 and virus or CR2 and virus for 1 hour on ice follow by three washes to remove unbound material and the cell-associated fluorescence was measured by lysing the cells and virus with 1% Triton X-100.

Results

Effect of Antibodies on Virus Binding and Infectivity of B Lymphocytes

Of the four monoclonal antibodies utilized in this work, two of them, 72A1 and F-2-1, have been previously reported to neutralize virus infectivity in the absence of complement (Hoffman et al., 1980; Strnad et al., 1982). Neutralization of virus infectivity is measured by the ability of the antibody to prevent the transforming strain of virus, MCUV5, from inducing immunoglobulin synthesis by human peripheral B cells in culture. The data in Table 6-1 show the extent of neutralization of virus with 72A1 and F-2-1, and also the effect of two non-neutralizing antibodies, E1D1 and E8D2. It was then important to compare the ability of the two neutralizing antibodies to inhibit virus binding as this would be an effective means by which virus could be neutralized. The non-neutralizing antibody E8D2 had no inhibitory effects on binding (Table 6-2). In contrast, the ability of 72A1 to neutralize virus infectivity can be accounted for by its ability to block virus binding. The observation that antibody F-2-1 failed to block virus attachment despite its ability to neutralize infectivity suggested that it blocked replication at a point following virus attachment.

Effect of Antibodies on Fusion of Virus Bound to Raji cells

Preincubation of R_{18}-labeled P3HR1-Cl13 virus with the nonneutralizing antibodies E8D2 and E1D1 had no effect on the amount of R18-labeled virus that bound to Raji cells, nor on the relief of self-quenching of the probe after cells were warmed to 37°C (Figure 6-1). In contrast, preincubation with antibody F-2-1 effectively inhibited the relief of self-quenching, even though the antibody did not affect binding of the labeled virus. These data suggested that the neutralization of virus

Table 6-1. Effect of antibodies F-2-1, E1D1, 72A1, and E8D2 on the ability of MCUV5 virus to induce immunoglobulin synthesis by fresh T-depleted human leukocytes.

Monoclonal antibody	Monoclonal:normal rabbit antibody (ng per culture)	Immunoglobulin conc. (ng/ml) with;		% inhibition
		antibody alone	antibody + virus	
F-2-1	1000:0	426	5958	62
	300:700	256	9396	41
	100:900	268	15628	1
	30:970	447	17862	0
	10:990	329	17696	0
E1D1	1000:0	151	14998	5
	300:700	120	15963	0
	100:900	224	17583	0
	30:970	132	17887	0
	10:990	144	15656	1
72A1	1000:0	208	686	96
	300:700	318	2429	85
	100:900	404	7604	52
	30:970	232	10303	35
	10:990	325	16632	0

E8D2	1000:0	199	15250	4
	300:700	316	14821	6
	100:900	352	16487	0
	30:970	124	17775	0
	10:990	140	17379	0
None	0:1000	263	15840	0

Table 6-2. Effect of antibodies F-2-1, 72A1, and E8D2 on the ability of [^3H] EBV to bind to receptor positive cells.

Antibody concentration (ug/ml)	[a]Total acid precipitable counts in presence of:		
	72A1	F-2-1	E8D2
600	177	5831	4891
400	162	5163	5080
200	187	5899	5832
100	220	5044	5379
50	417	5549	5682
25	1183	5038	5297
12.5	2296	4760	5494
None	4764		

[a]Counts bound to receptor negative cell= 110

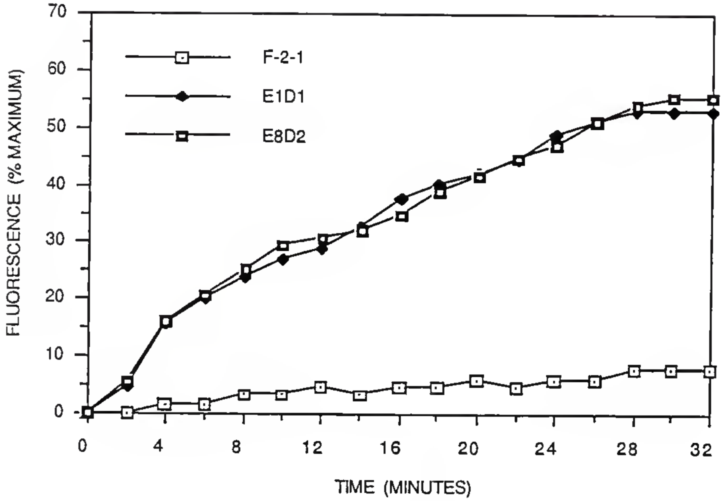


Figure 6-1. Effect of preincubation with 100ug of monoclonal antibodies on relief of self-quenching of R_{18}-labeled P3HR1-Cl13 virus added to Raji cells. Relief of self-quenching expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

infectivity by F-2-1 might be due to interference with the ability of the virus to fuse with the cell membrane.

Effect of Antibodies on Fusion on Virus Bound to T-depleted Leukocytes and BAT Cells

In order to rule out the possibility that the effects of the antibody were unique to the P3HR1-Cl13 strain of virus, the experiment was repeated with R_{18}-labeled MCUV5 virus and T-depleted human leukocytes. Of the three antibodies tested, only F-2-1 influenced the relief of self-quenching and the inhibition seen with F-2-1 was dose dependent (Table 6-3).

Although it was unlikely that the effects of the antibodies would be different from the EBV-transformed BAT cell line, these cells were also tested in assays with the antibodies. Figure 6-2 shows the results of the antibodies with BAT cells. Once again, the F-2-1 antibody inhibited fusion of bound virus with cells.

Specificity of Virus Binding to Epithelial Cells

Although previous work with the R_{18}-labeled EBV had indicated that the labeling had no effect on the specificity of binding to lymphocytes, and virus binding seemed to be confined to a subset of epithelial cells, there was still concern that the mild trypsinization of the epidermal sheets shortly before use might make them more non-specifically sticky for virus. To investigate this possibility, specificity was judged by two criteria, namely the ability of unlabeled virus to compete for binding of R_{18} labeled material, and the ability of the antibody 72A1, which inhibits virus binding to B cells, to inhibit R_{18} labeling of epithelial cells. The results are presented in Table 6-4 Raji cells were included as a comparative control. The figures in parentheses indicate

Table 6-3. Effect of antibody on the relief of self-quenching of R_{18}-labeled virus added to T-depleted leukocytes.

Antibody	Amount added (ug)	Fluorescence (% maximum)
F-2-1	100	6.1
	50	4.7
	25	5.6
	12.5	6.1
	6.25	9.4
	3.13	13.3
	1.56	24.3
E8D2	100	33.1
E1D1	100	29.7
None	0	31.1

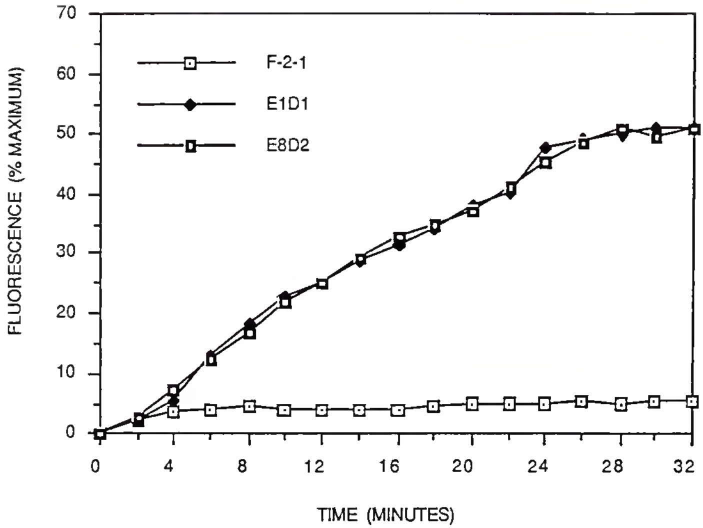


Figure 6-2. Effect of preincubation of virus with monoclonal antibodies on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to BAT cells. Relief of self-quenching is expressed as a percent of the maximum release obtained after addition of Triton X-100 (infinite dilution).

Table 6-4. Effect of antibody or unlabeled virus on binding of R_{18}-labeled virus.

Treatment	Relative amounts of virus bound to:		
	Parabasal cells	Basal cells	Raji
none	100 (285)[a]	100 (535)	100 (1076)
unlabeled virus	14 (41)	10 (54)	3 (35)
antibody 72A1	48 (137)	54 (290)	7 (73)

[a]Figures in parentheses indicate maximum fluorescence in arbitrary units.

maximum fluorescence obtained with each cell type after addition of Triton X-100. For comparison within cell type, this value was then given a score of 100. As expected, more virus actually bound to Raji cells, which are known to have a high density of receptors, than to any other cell type. As previously noted, almost twice as much virus bound to basal cells than parabasal cells despite the decreased reactivity of HB5 and B2 antibody with this population assayed in parallel. Unlabeled virus markedly reduced the amount of R_{18} label bound to each cell type, suggesting that little virus bound non-specifically to cells. In contrast, however, there was a significant difference between the ability of antibody to block attachment of virus to Raji cells and its ability to block attachment to epithelial cells.

Virus Binding in Presence of Soluble CR2

The extracellular domain of the receptor CR2 is composed of 15-16 short consensus repeat elements (SCR) that consist of 60-75 amino acids each. Analysis of CR2 deletion mutants and chimeric receptors of CR2 and CR1 have determined that the binding site of EBV and C3dg are within the first two amino-terminal SCRs (Lowell et al., 1989). The first two amino terminal SCRs of CR2 were expressed in the Baculovirus expression system by Nemerow et al. (1990). This protein is able to block infection of B lymphocytes by blocking binding of virus. In order to investigate further the difference in the ability to inhibit virus binding to epithelial cells with an anti-viral antibody, virus binding in the presence of soluble virus receptor was evaluated, the results are presented in Table 6-5. For Raji cells, 20ug of CR2 was able to inhibit virus binding by 90%, which parallels the effectiveness of the 72A1 antibody for inhibiting virus binding to these cells. The inhibition of virus binding to basal epithelial cells was 54% of the control in one experiment and 77% of the control in another

Table 6-5. Effect of soluble CR2 or 72A1 on binding of R_{18}-labeled virus.

Treatment	amount of R_{18}-EBV bound[a]		% inhibition of binding[b]	
	Raji	Basal	Raji	Basal
control	1090	858	0	0
CR2	118	398	90.2	54.6
72A1	119	463	90.1	46.0

[a]Fluorescence intensity in arbitrary units.
[b]Amount bound divided by amount bound to control sample.

experiment. The inhibition by the antiviral antibody 72A1, assayed in parallel, was comparable to the inhibition by 20ug of CR2.

Effect of Antibodies on Fusion of Virus Bound to Epithelial Cells

The antibody 72A1 failed to block virus binding to parabasal and basal epithelial cells to the same extent as it could block binding to T-depleted lymphocytes or Raji cells. This residual virus binding was evaluated for its ability to fuse with the cell membrane as indicated by fluorescence dequenching of the R_{18}-labeled EBV. Figure 6-3 shows that the virus that bound to the cells after incubation with 72A1 was unable to fuse with the cell membrane.

The monoclonal antibody F-2-1 which recognizes another envelope glycoprotein gp85 and inhibits infectivity of B lymphocytes without having any effect on virus binding was also tested in the fusion assay with epithelial cells. In contrast to what has been found with B cells, this antibody does not inhibit virus binding or fusion with epithelial cells (Figure 6-4). Another antibody that recognizes gp85, but is not a neutralizing antibody, E1D1, was also included in this study and this antibody had no effect on virus binding and fusion with epithelial cells (Figure 6-5).

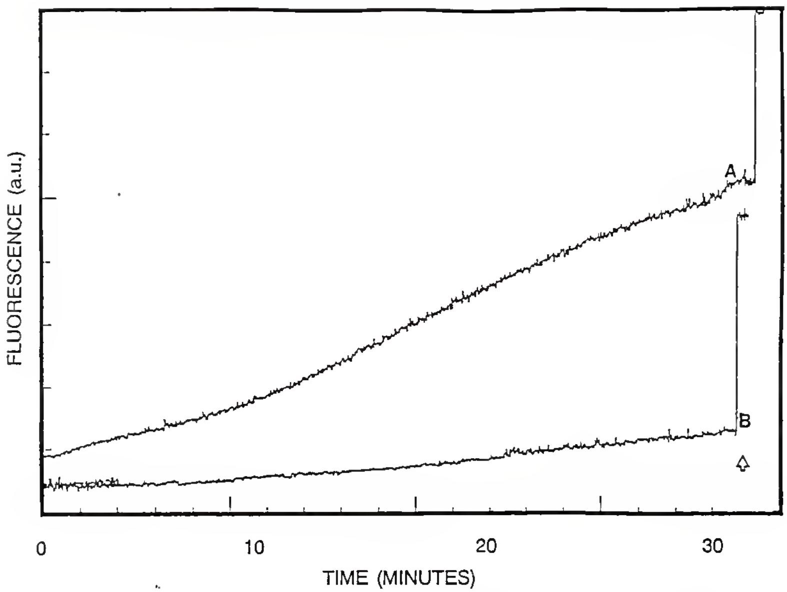


Figure 6-3. Effect of preincubation with 100ug of normal mouse immunoglobulin (A) or antibody 72A1 (B) on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to basal epithelial cells. Increase in fluorescence is expressed in arbitrary units (a.u.). Arrow indicates point at which Triton X-100 was added to measure maximum relief of self-quenching of bound virus (infinite dilution).

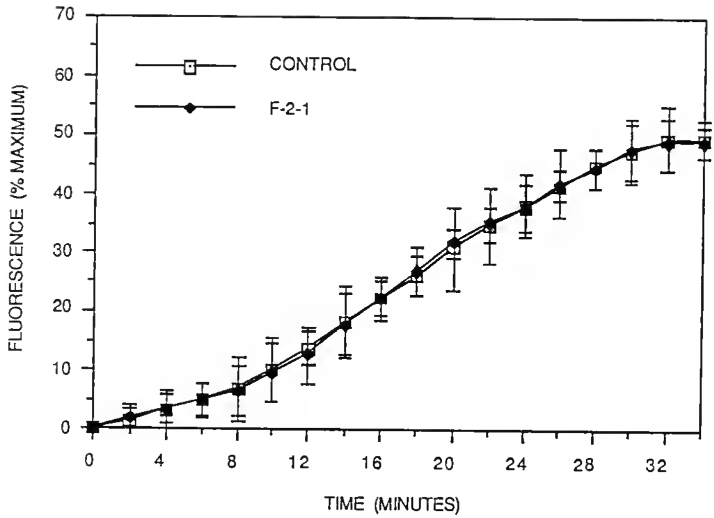


Figure 6-4. Effect of preincubation with 100ug of normal mouse immunoglobulin or antibody F-2-1 on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to basal epithelial cells. Increase in fluorescence is expressed as a percent of the maximum release after addition of Triton x-100 (infinite dilution). The vertical lines indicate the standard deviation of the mean of 4 experiments.

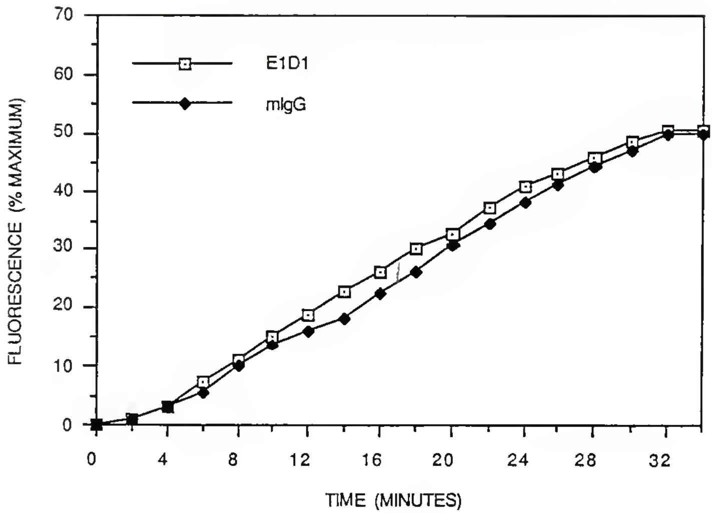


Figure 6-5. Effect of preincubation with 100ug of normal mouse immunoglobulin or antibody E1D1 on relief of self-quenching of R_{18}-labeled MCUV5 virus bound to basal epithelial cells. Increase in fluorescence is expressed as a percent of the maximum release after addition of Triton x-100 (infinite dilution).

Discussion

The initial observation that the monoclonal antibody F-2-1 was able to neutralize virus infectivity of B lymphocytes without inhibition of virus binding, suggested that the glycoprotein gp85 might be involved in events occurring after virus binding. The data presented in this chapter suggest that gp85 is involved in fusion of virus and B lymphocyte cell membranes. This antibody was tested with normal B cells, recently EBV-transformed cells and lymphoblastoid cells that have been in culture for many years. Fusion of virus with all three of these cell types was inhibited by this antibody against gp85. The gene encoding gp85 has been mapped to the BXLF2 open reading frame of EBV DNA (Heineman et al., 1988; Oba and Hutt-Fletcher, 1988). The sequence encoded a relatively hydrophobic protein that has homology with genes encoding herpes simplex type (HSV) 1 glycoprotein gH, varicella zoster (VZV) gpIII and glycoprotein p86 of human cytomegalovirus (CMV) (Cranage et al., 1988). Antibodies to each of these proteins neutralize virus in the absence of complement and antibodies to both HSV gH and VZV gpIII have been shown to block cell fusion by syncytial virus strains (Gompels and Minson, 1986; Keller et al., 1987). Thus EBV gp85 appears to be one of a family of homologous herpesvirus glycoproteins whose role in infectivity may be one of membrane fusion. Studies with purified recombinant gp350 and gp220 have implicated these two proteins in the internalization process (Tanner et al., 1987). Polyacrolein beads 0.9 um in diameter coated with the recombinant proteins bound to the EBV receptor on normal B cells and twenty-four hours later 10% of the beads were found to be discharged free into the cytoplasm. It is possible however that the discharge of gp350/220 coated beads into the cytoplasm of B cells does not completely reflect the

normal infectious process. Our data showing inhibition of relief of self-quenching of R_{18}-labeled virus by the F-2-1 antibody and the failure of the E1D1 antibody to influence fusion is evidence for involvement of gp85 in fusion of virus with the cell membrane, possibly leading to virus penetration. These results have been further investigated by comparing the ability of virosomes, which are liposomes with virus proteins incorporated, to bind and fuse to receptor positive cells (Haddad and Hutt-Fletcher, 1989). Virosomes were labeled with R_{18}, and were shown to behave in a manner similar to that of labeled virus. Monoclonal antibodies that inhibited binding of fusion of virus inhibited binding and fusion of virosomes, and virus competed with virosomes for attachment to cells. Virosomes made from virus proteins depleted of gp85 by immunoaffinity chromatography remained capable of binding to receptor positive cells but failed to fuse. These results are compatible with the hypothesis that gp85 is actively involved in the fusion of EBV with B lymphocytes and suggest that the ability of the antibody F-2-1 to neutralize infectivity of EBV represents a direct effect on the function of the envelope glycoprotein gp85 as a fusion protein. Virus entry into epithelial cells is apparently different from that with B cells. The antibody F-2-1 and the antibody E1D1 were not able to inhibit virus binding or fusion with epithelial cells. This contrast between epithelial cell fusion and B lymphocyte fusion was not the only difference found between these two cell types. The antibody 72A1, which recognizes the major viral envelope proteins gp350 and gp220, completely inhibits virus binding to B lymphocytes, but was unable to inhibit more than 50% of the virus bound to epithelial cells. However, the remaining virus that was bound to the epithelial cells was unable to fuse as indicated by the fluorescence dequenching of the bound virus.

The specificity of virus binding to epithelial cells was evaluated by the ability of unlabeled virus to compete for binding of R_{18}-labeled material. Unlabeled virus markedly reduced the amount of virus binding, whereas the antibody 72A1 was unable to inhibit all virus binding. Virus binding in the presence of soluble receptor was the next approach investigated and this reagent was also unable to completely inhibit virus attachment of virus to epithelial cells. The residual virus binding to epithelial cells is apparently non-functional in terms of the ability of the virus to fuse with the cell membrane as measured by the fluorescence dequenching assay. This binding may be irrelevant to the infectious cycle in this cell type but it could also be an indication that there is more than one type of binding interaction that is necessary for virus fusion to occur with the epithelial cell and that the antibody 72A1 is capable of preventing one of the interactions to an extent that fusion is inhibited.

The ability of a peptide corresponding to the amino terminus of gp350/220 to block virus binding to B cells indicates that this region is primarily responsible for virus attachment to B cells via CR2 (Nemerow et al., 1989). This peptide has a homology with the C3d protein, suggesting that a single domain may be used for binding of both ligands. This does not eliminate the possibility that other regions of gp350/220 also mediate or contribute to receptor interaction. It is once again relevant to point out that the anti-CR2 monoclonal antibody OKB7 was able to block binding of EBV and C3d with CR2 on B cells, but that OKB7 did not react with epithelial cells.

Considering the complexity of the EBV envelope and the specific tropism that the virus maintains, it is conceivable that certain envelope proteins may have functions that are unique to one type of host cell.

CHAPTER 7
SUMMARY AND CONCLUSIONS

Recapitulation

It is clearly evident that in order to understand the biologic activity of EBV, one must understand how this virus infects cells, and what components, such as surface membrane proteins are important to the process. The studies described here have focused on establishing an assay which measures membrane fusion of EBV with host cells in order to analyze the early events of Epstein-Barr virus infection. The viral envelope of herpesviruses are more complex than viruses such as influenza, vesicular stomatitis, Sendai, and Semliki Forest virus and it is likely that several herpesvirus glycoproteins are involved in virus entry. Epstein-Barr virus is unique in that it exhibits specific tropism for two target cells types, lymphocytes and epithelial cells, which provide discrete and distinct opportunities to study the complexities of virus entry.

Over recent years, the octadecylrhodamine (R_{18}) fluorescence dequenching assay has become widely used and accepted as a means of monitoring membrane fusion. The quantitation of membrane fusion by lipid mixing between labeled and non-labeled membranes resulting in dilution of R_{18} is an indirect approach, but it is a current technique of choice in membrane research.

The work in this dissertation has shown that the entry of EBV into lymphocytes and epithelial cells occurs independent of triggering by exposure to a low pH environment. In confirmation of previous studies with electronmicroscopy, virus entry

into the lymphoblastoid cell line Raji occurred at the plasma membrane and virus was endocytosed into fresh B cells and recently transformed cells. Virus that was endocytosed was exposed to low pH as indicated by the results of fusion assays with virus labeled with a pH sensitive fluorescent probe, but low pH was not a requirement of the fusion event. The effect of chloroquine on virus fusion was attributed to inhibition of endocytosis. Virus entry into epithelial cells occurred at the surface of the cells as indicated by the lack of inhibition of virus fusion under conditions when endocytosis was inhibited and also by measuring virus fusion with a pH sensitive probe.

The EBV receptor on B lymphocytes has been shown to be the CR2 protein. A monoclonal antibody to this protein, HB5, was used to determine the presence of a CR2-related receptor on epithelial cells. Basal epithelial cells expressed a CR2-related molecule that was present in approximately 20% of the population as judged by reactivity with the antibody HB5. Although almost all epithelial cells that bound virus were found in the HB5 positive population, there was not good correlation between expression of the epitope and the amount of virus binding. The epitope recognized on gp350/220 by antibody 72A1 was involved in binding of some, but not all virus binding to epithelial cells, and was possibly involved in virus fusion with this cell type. These results were in contrast with those with B lymphocytes for which the antibody 72A1 was capable of completely inhibiting virus binding. A monclonal antibody to an additional virus envelope glycoprotein, gp85, was shown to inhibit virus fusion with lymphocytes, but had no effect on virus fusion with epithelial cells. This finding suggests that gp85 has an active role in fusion of EBV with B lymphocytes, but this is not necessarily true for fusion with epithelial cells.

Importance of Present Studies and Future Directions

These studies have determined the mechanism of virus entry into B lymphocytes and epithelial cells. The contribution of two viral envelope glycoproteins, gp350/220 and gp85, in the fusion process has been evaluated. Future experiments with recombinant gp85, gp350, and gp220 proteins incorporated into liposomes will allow analyses of the functional roles of these proteins with the two target cell types. It will be important to determine if gp350 and gp220 have separate functions in epithelial cell and B lymphocytes and whether gp85 is dispensible for fusion of EBV with epithelial cells.

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BIOGRAPHICAL SKETCH

The candidate, Nancimae Miller, graduated in 1980 from Mother McAuley Liberal Arts High School in Chicago, Illinois. She earned a Bachelor of Science degree majoring in medical technology from Rush University at Rush-Presbyterian-St. Luke's Medical Center in Chicago in 1985. Her graduate work at the University of Florida began in 1985 as a student in the Department of Immunology and Medical Microbiology from which she received a Master of Science degree in 1988. She became a Ph.D. candidate in 1988 in the same department. Her research was conducted under the excellent guidance of Dr. Lindsey Hutt-Fletcher in the Department of Comparative and Experimental Pathology. She plans to pursue additional research training working on herpes simplex retinitis as a postdoctoral fellow in the laboratory of Dr. Sally Atherton at the University of Miami.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lindsey Hutt-Fletcher

Lindsey M. Hutt-Fletcher, Chair
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sue Anne Moyer

Sue Anne Moyer
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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